SWITCHGRASS YIELD, NUTRIENT UPTAKE, AND RHIZOSPHERE MICROBIAL COMMUNITY COMPOSITION AS AFFECTED BY CULTIVAR AND SOIL FERTILITY

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Dedication

This thesis is dedicated to my mother, Barbara Sawyer and my husband, Joseph Heinz, as well as our soon-to-be-born child - we are eagerly anticipating your arrival within weeks of my official PhD completion. Cheers to great accomplishments and new beginnings!

Abstract

Switchgrass (*Panicum virgatum* L.) is a native warm-season perennial that can be grown as a bioenergy crop on fragile or low-productivity soils, reserving prime agricultural land for food crops. The objectives of this dissertation were to evaluate switchgrass and mixed native perennial production and nutrient removal as a function of nitrogen (N) and phosphorus (P) application, harvest regime, and cultivar selection at two contrasting locations in Minnesota. Because bioenergy crop management decisions may alter rhizosphere microbial populations and associated ecosystem functions such as carbon and N cycling, this research also incorporated next-generation sequencing technology to examine bacterial and fungal community composition as a function of switchgrass cultivar selection as well as N and P fertility.

The first experiment evaluated biomass production and N removal in switchgrass and mixed native perennials as a function of harvest regime (anthesis and post-frost) and N application rate (0, 56 and 112 kg N ha^{-1}) at two locations in Minnesota. One plot was located near Becker, Minnesota, on Hubbard-Mosford complex loamy sand (Sandy, mixed, frigid Entic (Hubbard) and Typic (Mosford) Hapludolls) and the other was located near Lamberton, Minnesota, on Amiret-Swanlake loams (fine-loamy, mixed, superactive, mesic Calcic Hapludolls (Amiret) and fine-loamy, mixed, superactive, mesic Typic Calciudolls (Swanlake)) with 3-6% slope. Two switchgrass forage varieties, 'Shawnee' and 'Sunburst', produced 8.1 and 28.2 Mg ha^{-1} over three post-establishment years at Becker and Lamberton, respectively, and a low-diversity grass mix was similar in production to 'Shawnee'. 'Liberty' switchgrass, a new bioenergy variety, yielded less

than ‘Sunburst’, ‘Shawnee’, and the low-diversity grass mix. Based on these results, recommendations for maximum biomass production include planting either a well-adapted switchgrass variety or low-diversity grass mix fertilized with 56 kg N ha^{-1} annually, post-establishment, on loam soil, or with 112 kg N ha^{-1} in split application on sandy loam soil, and utilizing a post-frost harvest regime to remove less N and promote stand longevity over time.

The second experiment, conducted on the anthesis harvest plots described in the first experiment, examined the effects of cultivar and N fertilization on bacterial community composition in the rhizosphere of ‘Sunburst’, ‘Shawnee’, and ‘Liberty’ switchgrass. While rhizobacterial community structure was primarily shaped by the existing microbial inoculum in the soil, variability in community composition was evident in response to cultivar and N. Only N fertilization, however, resulted in differences at both locations that were consistent across bacterial orders, including orders containing genera involved in N dynamics in soil: *Nitrosomonadales* and *Rhodocyclales*.

The third experiment was located near Lamberton, Minnesota, on Webster clay loam (Fine-loamy, mixed, superactive, mesic Typic Endoaquoll). This experiment examined the effects of cultivar and P fertilization (0, 19.6, 35.1, and 58.6 kg P ha^{-1}) on biomass yield, phosphorus removal, and rhizosphere microbial community structure in switchgrass grown on a low to medium soil test P soil. Post-frost biomass yields on low to medium soil test P soils increased linearly in response to P applied prior to establishment. ‘Shawnee’ produced greater biomass than ‘Liberty’ or ‘Sunburst’ (11.3, 10.2, and 8.6 Mg ha yr^{-1}, respectively) over three years. Bacterial and fungal community

structure was influenced by cultivar, but not P, although existing soil characteristics explained a greater proportion of variability in the rhizosphere community composition than did treatment effects.

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Introduction

Switchgrass (*Panicum virgatum* L.) is considered a desirable bioenergy crop based on its broad geographic range, high yield potential on marginal lands, and ability to be managed with conventional hay-making machinery (Vogel et al. 2002; Mitchell et al. 2008). Perennial polycultures of native plants may also yield as much, if not more, biomass relative to perennial monocultures (Mangan et al. 2011; Jungers et al. 2015a). Regardless of species combination, producers need cultivar, fertilization, and harvest options for best management practices. Production systems that utilize beneficial soil microbes will also be increasingly necessary (Arenz et al. 2014), particularly in areas where fertilizer application may risk water quality degradation, such as near water bodies or on excessively-drained soils.

Switchgrass upland and lowland ecotypes are each adapted to different edaphic and climatic conditions. Upland ecotypes, found in areas not prone to flooding, are adapted to northern latitudes, while lowland ecotypes, adapted to floodplains, generally have poor winter survival in northern regions (Vogel 2000; Casler et al. 2004). Only recently has the first biomass-type switchgrass become available: 'Liberty', released in 2014, is a lowland ecotype bred for biomass yield and winter hardiness (Vogel et al. 2014). Because several studies have demonstrated inconsistent yields among switchgrass cultivars as a result of cultivar x environment interactions (Casler and Boe 2003; Berdahl et al. 2005; Parrish and Fike 2005), further evaluation of 'Liberty' biomass yield potential is warranted in northern environments, particularly in comparison to productive forage switchgrass cultivars such as 'Shawnee' or 'Sunburst'.

Maximizing nitrogen (N) and phosphorus (P) fertilizer use efficiency is essential for economically viable and environmentally responsible production of perennial grass biomass. Switchgrass and other warm-season grasses generally respond positively to N fertilization (e.g. Muir et al. 2001; Vogel et al. 2002; Mulkey et al. 2008), but ideal N application rates vary as a function of site yield potential, cultivar selection, and harvest management (Parrish and Fike 2005; Mitchell et al. 2008). Understanding P needs in switchgrass is also essential, because inadequate P can have adverse affects on germination and biomass yield (Morris et al. 1982; Sutton et al. 1983; Kering et al. 2012). However, the effect of P fertilization is often confounded by the symbiotic relationships formed with arbuscular mycorrhizal (AM) fungi that enhance the ability of warm-season grasses to grow on low-P soils (Hetrick et al. 1990; Brejda et al. 1993).

Soil microorganisms can enhance crop growth by performing essential biogeochemical transformations such as organic matter decomposition and nutrient mineralization, and they utilize a number of direct and indirect plant growth promotion mechanisms such as antibiosis and improved nutrient uptake (Glick 1995; Dobbelaere et al. 2003; van Loon 2007). The composition of the soil microbial community is shaped by both soil properties and plant characteristics. In agricultural systems, communities are also greatly influenced by practices such cultivar selection and fertilizer application (Germida and Siciliano 2001; Berg and Smalla 2009; Inceoğlu et al. 2012; Philippot et al. 2013). Improved understanding of rhizosphere microbial communities in bioenergy cropping systems such as switchgrass is essential to developing sustainable production, particularly when utilizing marginal land (da Jesus et al. 2010; Liang et al. 2016).

The objectives of this dissertation were to evaluate switchgrass and mixed native perennial production and nutrient removal as a function of N and P fertilizer application, harvest regime, and cultivar selection at two locations in Minnesota. Because bioenergy crop management decisions may alter rhizosphere microbial populations and associated ecosystem functions such as carbon and nitrogen cycling, this research also incorporated next-generation sequencing technology to examine bacterial and fungal community composition as a function of switchgrass cultivar selection as well as N and P fertility.

Chapter 1: Switchgrass and mixed perennial biomass production as affected by nitrogen fertility and harvest management

The objective of this study was to determine the effects of N fertilization and two harvest regimes on biomass yield and N removal in switchgrass monocultures and perennial polycultures on two marginal productivity soils. 'Liberty', the first bioenergy switchgrass variety, was compared in productivity to two hardy forage varieties, 'Shawnee' and 'Sunburst' switchgrass, and three perennial prairie polycultures: a low-diversity grass mix, a grass/legume mix, and a high-diversity grass/forb/legume mix, representative of plant hardiness zone 4.

Chapter 2: Rhizobacteria community structure as a function of cultivar and nitrogen in switchgrass grown on two marginal soils

The objective of this study was to examine the effects of cultivar, N fertilization and soil physiochemical parameters on rhizosphere bacterial community structure in 'Liberty', 'Shawnee', and 'Sunburst' switchgrass using Illumina amplicon sequencing of the 16S rRNA gene. We selected two contrasting locations for this experiment, each representing conditions under which bioenergy crop production may be economically and logistically feasible: a sloping, eroded loam with 4.9% organic matter and an excessively drained loamy sand with 1.3% organic matter.

Chapter 3: Cultivar and phosphorus fertilization effects on switchgrass biomass yield, phosphorus removal, and rhizosphere microflora

The objectives of this study were twofold: 1) to evaluate biomass yield and P removal as a function of P fertilizer application rate in 'Liberty', 'Shawnee', and 'Sunburst' switchgrass on a low to medium soil test P loam soil, and 2) to examine the rhizosphere microbial community response to cultivar, P fertilization, and soil/plant physiochemical parameters using Illumina amplicon sequencing of the 16S rRNA gene in bacteria and of the ITS1 (internal transcribed spacer) region in fungi over two years.

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Chapter 1 – Switchgrass and mixed perennial biomass production as affected by nitrogen fertility and harvest management

Abstract

Biofuel production using native perennials on marginal soils can reduce U.S. dependence on foreign oil and curtail greenhouse gas emissions without diminishing food crop production. In this research, we quantified biomass production and nitrogen (N) removal as a function of harvest regime (anthesis and post-frost) and N application rate (0, 56 and 112 kg N ha^{-1}) on two marginal sites in Minnesota. We examined three switchgrass (*Panicum virgatum* L.) monocultures, including 'Liberty', a new bioenergy variety, and three polycultures: grass-only, grass-legume, and grass-legume-forb. At Becker, post-frost harvest yields totaling 11.0 Mg ha^{-1} over three years were achieved in 'Sunburst' and 'Shawnee', while 'Liberty' produced 7.0 Mg ha^{-1} when fertilized at 112 kg N ha^{-1} yr^{-1}. At Lamberton, post-frost harvest yields in 'Shawnee', 'Sunburst', and 'Liberty' totaled 32.5, 29.9 and 21.2 Mg ha^{-1} respectively, over three years, when fertilized at 56 kg N ha^{-1} yr^{-1}. Yields of the low-diversity (LD) grass mix were similar to 'Shawnee' switchgrass at both locations. Yield differences between harvest regimes varied by cultivar and location, although most feedstocks produced similar or greater yields in the post-frost harvest. Our results indicate that maximum biomass production can be achieved with either a well-adapted switchgrass variety or LD grass mix fertilized with 56 kg N ha^{-1} annually, post-establishment, on a moderately-productive loam soil, and with 112 kg N ha^{-1} annually on an excessively-drained sandy soil. While producers may have flexibility in harvest timing

for some feedstocks in the first few years following establishment, a post-frost harvest regime will remove less N and promote stand longevity with fewer inputs over time.

Introduction

The *Energy Independence and Security Act of 2007*, under full implementation, will require production of 79 billion L yr^{-1} of bio-based transportation fuel from sources other than corn (*Zea mays* L.) grain by 2022. A major challenge facing producers, however, is to dramatically increase biomass feedstock supply without reducing food crop production or causing adverse environmental impacts from land use change (Tilman et al. 2009). Marginal lands – those that are poorly suited to annual row crops as a result of climatic or edaphic limitations, or those that pose environmental risks such as erosion (Gelfand et al. 2013) – can be used to produce perennial biomass feedstock for biofuels (Varvel et al. 2008; Gelfand et al. 2013).

Switchgrass (*Panicum virgatum* L.) is considered a desirable bioenergy crop based on its broad geographic range, high yield potential on marginal lands, and ability to be managed with conventional hay-making machinery (Vogel et al. 2002; Mitchell et al. 2008). Switchgrass has upland and lowland ecotypes, each adapted to different edaphic and climatic conditions. Upland ecotypes are found in areas not prone to flooding and are adapted to northern latitudes. Lowland ecotypes are adapted to floodplains and generally occur at southern latitudes in the US, having poor winter survival in northern regions (Vogel 2000; Casler et al. 2004). Lowland ecotypes, however, generally produce greater biomass yields than upland ecotypes, and could enhance yields across North America with improved winter

hardiness (Vogel et al. 2014). Improving switchgrass yield for forage and hay has been the focus of research for more than 80 years (Vogel 2000), and several productive forage cultivars such as 'Alamo', 'Cave-in-Rock', and 'Shawnee' have been used in bioenergy feedstock research since the early 1990s (Mitchell et al. 2008; Casler and Vogel 2014). Only recently, however, has the first biomass-type cultivar become available. 'Liberty' switchgrass, released in 2014, is a lowland ecotype bred for biomass yield (Vogel et al. 2014). Yields of 'Liberty' averaged 18.1 Mg dry matter (DM) ha^{-1} compared with 12.5 Mg DM ha^{-1} for 'Shawnee', a highly productive forage type, over three post-establishment years in Nebraska (Vogel et al. 1996; Vogel et al. 2014). Yields were less reliable in Wisconsin, where 'Shawnee' produced more biomass than did 'Liberty' at two of three sites, averaging 12.3 compared to 10.2 Mg DM ha^{-1}, respectively (Vogel et al. 2014). Because several studies have demonstrated inconsistent yields among switchgrass cultivars as a result of cultivar x environment interactions (Casler and Boe 2003; Berdahl et al. 2005; Parrish and Fike 2005), further evaluation of 'Liberty' biomass yield potential is warranted in northern environments.

Switchgrass, however, is not the only viable bioenergy feedstock (Mitchell et al. 2008; Mulkey et al. 2008). Perennial polycultures of native plants may yield as much, if not more, biomass relative to perennial monocultures, although production depends on species mix, soil productivity potential, applied fertilizer, and harvest regime (Mangan et al. 2011; Jungers et al. 2015a; Duran et al. 2016). Polycultures reduce environmental risks inherent to relying on a single species, and polycultures may require fewer pesticides as a result of enhanced resistance to insect pests, plant disease, and exotic species invasion (Hill et al. 2006; Mulkey et al. 2008; Waramit et al. 2014). While several warm-season grasses and

perennial polycultures have been identified as potential biomass candidates, results from side-by-side comparisons of yield from polycultures and switchgrass monocultures are inconsistent (Griffith et al. 2011; Jungers et al. 2015a; Duran et al. 2016), and very few studies have also evaluated interactive fertility and harvest treatments.

Regardless of species combination, native perennial biomass plantings likely will be subject to low-fertility or other challenging edaphic conditions on marginal lands, and producers need fertilization and harvest options for best management practices. Switchgrass and other warm-season grasses generally respond positively to N (e.g. Muir et al. 2001; Vogel et al. 2002; Mulkey et al. 2008), but maximizing N fertilizer use efficiency is challenging (Brejda 2000) because ideal application rates are a function of site yield potential, cultivar, and harvest management (Parrish and Fike 2005; Mitchell et al. 2008). Results from N application rate studies in switchgrass managed for biomass production in the upper Midwest are wide-ranging. These include no yield response at 56 kg N ha^{-1} N in Wisconsin (Duran et al. 2016), maximum yield response at 56 kg N ha^{-1} in South Dakota (Mulkey et al. 2008) and maximum yield response at 140 kg N ha^{-1} in Iowa (Waramit et al. 2014). Even less information exists regarding best management practices and fertilizer response in mixed perennial prairie biomass, and management strategies implemented for a species grown in monoculture may not apply to polycultures (Jungers et al. 2015b; Duran et al. 2016). While nutrients removed through harvest will need to be replaced to avoid soil nutrient depletion, stand decline, and lower yields (Jungers et al. 2015b), caution must be observed to avoid N losses to the environment (Mitchell et al. 2008; Duran et al. 2016).

Therefore, maximizing N fertilizer use efficiency is essential for economically viable and environmentally responsible production of perennial grass biomass.

Management of bioenergy feedstocks also includes harvest timing, and harvest options for producers may vary according to species mix and location. In Nebraska, the anthesis harvest, defined as R3 to R5 stage, or panicles fully emerged to postanthesis (Moore et al. 1991), harvest can optimize biomass yield and may be most convenient for producers, as it occurs in late summer prior to annual row crop harvest (Vogel et al. 2002; Waramit et al. 2014). Anthesis harvests at northern latitudes may, however, jeopardize switchgrass stand persistence by depletion of carbohydrate reserves in late summer/early fall during regrowth (Casler and Boe 2003; Mulkey et al. 2006). Alternatively, a post-frost harvest may reduce N fertilizer needs because N is translocated to plant roots during fall senescence (Vogel et al. 2002; Mitchell et al. 2008). Information on ideal biomass production harvest management schedules is limited (Waramit et al. 2014) and in mixed perennial polycultures, harvest timing can also impact species mixture composition (Mulkey et al. 2008).

Our objective was to determine the effects of N fertilization and two harvest regimes on biomass yield and N removal in switchgrass monocultures and perennial polycultures on two marginal productivity soils. We examined ‘Liberty’ switchgrass, comparing productivity to ‘Shawnee’ switchgrass, ‘Sunburst’ switchgrass, and three perennial prairie polycultures: a low-diversity grass mix, a grass/legume mix, and a high-diversity grass/forb/legume mix, representative of USDA plant hardiness zone 4.

Materials and methods

Site description and experimental design

We conducted this experiment from 2012-2015 at the Sand Plain Research Farm in Becker, MN (45°32.32' N, 93°52.53' W) and from 2013-2016 at the Southwest Research and Outreach Center in Lamberton, MN (44°14.40' N, 95°19.00' W). Becker and Lamberton are located in USDA plant hardiness zones 4a and 4b, respectively, where average annual extreme minimum temperatures are between -34.4° to -31.7°C, and -31.7° to -28.9°C, respectively. Local climate data (Table 1-1) were obtained from NOAA's Cooperative Network, via the Midwestern Regional Climate Center, cli-MATE (MRCC Application Tools Environment, http://mrcc.isws.illinois.edu/CLIMATE/, accessed 4/2016). The 30-year (1981-2010) normal temperatures are 5.9 and 7.0°C, with average minimum winter temperatures (December-February) of -14.6 and -13.3°C at Becker and Lamberton, respectively. The 30-year normal precipitation at Becker and Lamberton is 704 and 714 mm, respectively, with 512 and 519 mm falling during the May – October growing season.

At Becker, the 0.8-ha site was located on Hubbard-Mosford complex loamy sand (Sandy, mixed, frigid Entic (Hubbard) and Typic (Mosford) Hapludolls). This soil is considered marginal in productivity because of excessive drainage (class 4s). Our 0.8-ha site at Lamberton was on Amiret-Swanlake loams (fine-loamy, mixed, superactive, mesic Calcic Hapludolls (Amiret) and fine-loamy, mixed, superactive, mesic Typic Calciudolls (Swanlake)), 3-6% slope, considered marginal in productivity because of erosion (class 2e). At Becker, pre-plant soil characteristics were as follows: 25.3 mg kg^{-1} P (Bray P1), 53.0 mg kg^{-1} K, 1.3% soil organic matter (SOM), 6.4 pH. Pre-plant amendments were broadcast and

incorporated: 67 kg ha^{-1} triple superphosphate (TSP, 0-45-0), 168 kg ha^{-1} potassium magnesium sulfate (KMS, 0-0-22-18), and 247 kg ha^{-1} potassium chloride (KCl, 0-0-60) based on University of Minnesota Recommendations (Kaiser et al. 2011b). At Lamberton, pre-plant soil characteristics were as follows: 13.0 mg kg^{-1} P (Bray P1), 140.0 mg kg^{-1} K, 4.9% SOM, 5.3 pH. No pre-plant amendments were applied at Lamberton because nutrient status was adequate for native grass production.

The experimental design was a split, split-plot, randomized complete block with four replications. Treatments included two harvest regimes, six feedstocks, and three N fertilizer rates. The main plot treatment was harvest regime, either within two weeks of average anthesis stage for switchgrass or after a killing frost. Subplots were biomass feedstocks composed of three switchgrass monocultures and three prairie perennial polycultures. The switchgrass cultivars were 'Shawnee', 'Sunburst', and 'Liberty'. 'Shawnee' is a hardy upland variety, bred for forage quality and yield through the USDA-ARS breeding program at Lincoln, Nebraska. 'Shawnee' was selected out of the 'Cave-in-Rock' switchgrass cultivar, whose germplasm originated in southern Illinois (Vogel et al. 1996; Tober et al. 2007). 'Sunburst' is an extremely hardy upland cultivar selected from seed originating near Yankton, in southeastern South Dakota. 'Sunburst' has yields similar to or greater than 'Cave-in-Rock' but also has superior germination, seedling vigor, and stand establishment (Boe and Ross 1998; Berdahl et al. 2005; Tober et al. 2007). 'Liberty' was bred specifically for bioenergy production and is the first lowland-type cultivar adapted to USDA plant hardiness zones 4, 5, and 6 (Vogel et al. 2014). The polyculture feedstocks included a low diversity grass mix (LD), the same low diversity grass mix plus three native legume species

(LD + legumes), and a 14-species, high diversity Conservation Reserve Program mix (CRP) adapted to Minnesota (Table 1-2). Sub-subplots were N fertilizer applied at 0, 56, 112 kg N ha^{-1}, at the start of the second and subsequent growing seasons in the form of urea coated with urease inhibitor NBPT (N-(n-butyl) thiophosphoric triamide) to minimize volatilization losses. Fertilizer N was applied in a single surface broadcast application at Lamberton and in two equal split surface broadcast applications at Becker to avoid leaching losses. Sub-subplots measured 1.8 m by 4.6 m.

Plot establishment and harvest

Plots were established in May of 2012 at Becker and May of 2013 at Lamberton. Plots were seeded using a Wintersteiger Plotmaster small plot grain drill (Wintersteiger Inc., Salt Lake City, Utah), into a firm, smooth seedbed at a depth of 6 to 13 mm in rows 15.2 cm apart. At Becker, the LD and LD + legume seeds were too lightweight to effectively pass through the planter, so those plots were seeded by hand. All plots were seeded to a minimum of 325 pure live seed (PLS) per square meter. During the 2012 establishment year, irrigation was applied at Becker to ensure experiment viability under extremely dry conditions (Table 1-3). Irrigation was discontinued after the first year. No irrigation was applied at Lamberton. No herbicides were used during the establishment year at Becker, but plots were mowed above grass height to eliminate weed seed heads in June 2012. Herbicides were used on the grass-only plots during the establishment year at Lamberton and in subsequent years at both sites, as outlined in Table 1-4. The polyculture plots at both sites were hand-weeded annually, as necessary. Plant percent cover, evaluated in late spring of the first two treatment

years, was based on the frequency grid method of Vogel and Masters (2001): presence or absence of desired species was counted within a randomly-placed metal grid containing 25 squares of 15 cm by 15 cm. The count was completed at least twice per plot, and percent cover was based on the number of species present.

Biomass was harvested using a Carter plot forage harvester (Carter Manufacturing Company, Inc., Brookston, Indiana) from the 0.9 by 4 m center of each plot to a 10 cm height. Harvested biomass was weighed in the field. Prior to mechanical harvest, two subsamples were hand-cut within each subplot. The subsamples were refrigerated at 10°C until processing, at which point they were separated into weed and planted species group components (grasses, legumes, and/or forbs), and the planted species components were weighed, dried at 50 °C in a forced-air oven for 72 hours, and reweighed to determine percent dry matter (DM) for each planted species group. After drying, subsamples were ground to pass a 2-mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ) and combined to obtain one sample per plot. Total N was determined by combustion, using a LECO Nitrogen Analyzer (LECO Corporation, St. Joseph, Michigan). Total biomass N removal was calculated using percent total N multiplied by biomass DM. Nitrogen use efficiency (NUE) was calculated as the difference between yield at 0 kg N ha^{-1} and 56 or 112 kg N ha^{-1}, divided by applied N.

Statistical analysis

Data were analyzed using mixed linear models (PROC MIXED) in the SAS software program, Version 9.4 of the SAS System for Windows (Copyright © 2002-2012, SAS

Institute Inc., Cary, North Carolina). All datasets met normality assumptions for analysis of variance. Replication was treated as a random effect; all other effects were treated as fixed. Significant differences, based on $\alpha = 0.05$, were determined using 'lsmeans' with the 'pdiff' option, and mean separation was done using the 'lines' option in PROC PLM in SAS (Appendix A). Each site was analyzed separately because of substantial differences in soil type and weather conditions.

Results and discussion

Total biomass yields

Overview of all treatments

At Becker, there was a harvest × feedstock × N fertilizer interaction effect on total biomass yield ($p < 0.001$), and at Lamberton, there were harvest × feedstock and feedstock × N fertilizer interaction effects on total biomass ($p = 0.001$, $p = 0.031$, respectively) (Table 2). Harvest interactions occurred largely as a result of lesser CRP yields in the post-frost harvest relative to the anthesis harvest, while all other feedstocks produced equal or greater yields in the post-frost relative to the anthesis harvest (Figures 1-1 and 1-2). The CRP mix contained > 20% cool-season grass seed by weight, which likely accounted for lesser post-frost yields; cool-season grasses mature, senesce, and deteriorate earlier in the season than do warm-season grasses. No other feedstocks contained cool-season grasses. Nitrogen fertilizer interactions were a result of variability in yield response magnitude among different feedstocks at each location. At Becker, 'Sunburst' produced more biomass at 112 than at 56 kg N ha^{-1} in the anthesis harvest, whereas 'Shawnee' and

LD produced more at 112 than at 56 kg N ha^{-1} in the post-anthesis harvest. 'Liberty', however, produced less at 112 than at 56 kg N ha^{-1} in the post-frost harvest, likely as a result of weed pressure (Figure 1-3). All other feedstock yields were equivalent at 56 and 112 N ha^{-1} in both harvests. At Lamberton, there were no yield gains in LD + legumes in response to N application, and 'Sunburst' yields were similar between the control and 56 kg N ha^{-1}. Yields of all other feedstocks increased in response to N fertilization relative to the control, but there were no yield differences between 56 and 112 kg N ha^{-1}.

Switchgrass monocultures

The highest-yielding monocultures for both harvest treatments at both sites were 'Shawnee' and 'Sunburst' switchgrass; however, some treatment interactions affected relative ranking. At Becker, yields for the two cultivars were similar at the same N rates, with the exception of 'Sunburst' yielding nearly 30% more dry matter (DM) than 'Shawnee' in the anthesis harvest at 112 kg N ha^{-1}. 'Shawnee' and 'Sunburst' were also similar to each other in percent ground cover at Becker (Table 1-6). At Lamberton, yields of 'Sunburst' and 'Shawnee' were similar when unfertilized, but yields of 'Shawnee' were 8.7% and 10.8% greater than 'Sunburst' when fertilized at 56 and 112 kg N ha^{-1}, respectively, despite having similar percent ground cover in both years. These results are not unexpected; Vogel et al. (2014) reported lower yields in N-fertilized 'Sunburst' relative to 'Shawnee' in Wisconsin, while Berdahl et al. (2005), found greater yields in N-fertilized 'Sunburst' relative to 'Shawnee' in North Dakota.

‘Liberty’ generally yielded less than ‘Shawnee’ and ‘Sunburst’ at both locations within harvest treatments. At Becker, the fertilized anthesis harvest yields of ‘Liberty’ (3.6 Mg DM ha^{-1}) were equivalent to the unfertilized yields of ‘Shawnee’ and ‘Sunburst’, and three times less than the anthesis yields of ‘Sunburst’ fertilized at 112 kg N ha^{-1} (11.3 Mg DM ha^{-1}). In the post-frost harvest treatment receiving 56 kg N ha^{-1}, ‘Liberty’ yield was equivalent to ‘Shawnee’ and ‘Sunburst’ (9.7 Mg DM ha^{-1}), but at 112 kg N ha^{-1}, ‘Shawnee’ and ‘Sunburst’ were nearly 60% greater than ‘Liberty’. ‘Liberty’ had lesser percent ground cover at Becker relative to ‘Shawnee’ and ‘Sunburst’, and ‘Liberty’ yields were adversely impacted by weed competition. Wet weed biomass in ‘Liberty’ was greater in ‘Shawnee’ and ‘Sunburst’ for all levels of N at the anthesis harvest and at 112 kg N ha^{-1} in the post-frost harvest. At Lamberton, the anthesis harvest of ‘Liberty’ (averaged over N treatments) was less than ‘Shawnee’ but similar to ‘Sunburst’, while the post-frost yield of ‘Liberty’ was less than both ‘Shawnee’ and ‘Sunburst’. These differences in yield were likely related to the greater post-frost wet weed biomass in ‘Liberty’ relative to ‘Shawnee’ or ‘Sunburst’ (Table 1-7), despite similar ground cover percentages between all three switchgrass cultivars. When averaged over harvest treatment, yields in unfertilized and fertilized ‘Shawnee’ and ‘Sunburst’ were similar to or greater than yields in fertilized ‘Liberty’. These results sharply contrast with those of Vogel et al.’s (2014) Nebraska trials, where yields of ‘Liberty’ were 31% greater than those of ‘Shawnee’ over three years, but are more consistent with their Wisconsin trials, where ‘Liberty’ produced less than ‘Shawnee’ at two of three sites (112 kg N ha^{-1} was applied annually at both locations).

Similar to other studies (Casler and Boe 2003; Berdahl et al. 2005), our results suggest that productivity and survival in each cultivar was influenced by location factors such as soil type, climate, and weed pressure. 'Liberty' may be less drought-tolerant than upland cultivars 'Shawnee' and 'Sunburst', which are generally adapted to drier conditions (Tober et al. 2007) such as those found at Becker. 'Liberty' suffered a 42% loss in ground cover at Becker between 2013 and 2014 following and extremely dry growing season (July through September) and below-average winter temperatures in January through March.

While stand productivity could decline with repeat anthesis harvests (Casler and Boe 2003), producers may have some flexibility in harvest timing, particularly with well-adapted cultivars such as 'Shawnee' or 'Sunburst' grown on loam soils. Similar to Stout and Jung (1995), we found that yields of well-adapted switchgrass cultivars are maximized with 112 kg N ha^{-1} on low-fertility, drought-prone sites such as Becker, while at moderate-fertility sites with sufficient water-holding capacity, such as Lamberton, 56 kg N ha^{-1} is optimum.

Perennial polycultures

At both locations, the highest-yielding polycultures produced equivalent or lesser amounts of biomass compared to the best-adapted switchgrass monocultures. The LD mix was the best-yielding polyculture overall and equivalent to 'Shawnee' under all circumstances except the anthesis treatment fertilized at 112 kg N ha^{-1} at Becker. These results are consistent with those of Jungers et al. (2015a) and Mangan et al. (2011), who

found similar yields for N-fertilized low-diversity grass mixtures and switchgrass monocultures in a post-frost harvest regime in Minnesota. Harvest treatment effects on LD mix yields were minimal. There were no yield differences between harvests at Lamberton, and at Becker, post-frost yield in LD mix was greater than anthesis yield only when fertilized at 112 kg N ha^{-1}. Weed pressure was also greater in the fertilized anthesis LD plots relative to the post-frost LD plots. While LD biomass yields increased in response to N fertilization relative to the control at both locations, increasing N from 56 to 112 kg ha^{-1} improved yields only in the post-frost harvest at Becker.

The addition of legumes to the low-diversity grass mix did not improve yields relative to the LD mix alone. At Becker, yields of LD + legumes were equivalent to or less than the LD mix for all treatments. There were no increases in LD + legume yield relative to the control as a function of N fertilization in the anthesis harvest. LD + legume yield did increase in response to N fertilization relative to the control in the post-frost harvest, but this increase was a result of increasing grass biomass; legumes accounted for less than 3% of post-frost biomass (Table 1-8). At Lamberton, yields of LD + legumes were less than for the LD mix under all circumstances, and there was no effect of N fertilization on biomass yields in the LD + legumes feedstock. While stands were similar between LD + legumes and LD at Becker, at Lamberton legumes may have competed with the grasses for resources in 2014, resulting in lesser grass establishment and biomass production in the LD + legumes relative to LD. By 2015, however, the difference in grass stand percentage had disappeared. These results suggest that there was no facilitation of grass growth via legume N fixation, contrary to results from Jungers et al. (2015a) and Posler et

al. (1993), who found legume facilitation in unfertilized grass + legume mixtures in Minnesota and Kansas, respectively. At Becker, the low native soil fertility should have allowed for legume facilitation, particularly in the anthesis harvest when legumes comprised 34.6% of total wet weight biomass in the control treatment. Legumes only accounted for 6.6% of total wet weight biomass in the unfertilized treatment for the post-frost harvest, however. Moisture-limiting conditions may have diminished any N facilitation effect, regardless of legume proportion. At Lamberton, N mineralization from organic matter may have minimized any effect of facilitation, even though legumes accounted for 22 and 10% total biomass in the anthesis and post-frost harvests, respectively. At both locations, legume biomass was larger in the anthesis relative to the post-frost harvest. Visual observations indicated that after senescence, the legumes dropped most of their leaves, resulting in a possible underrepresentation of total leguminous biomass.

The CRP mix was among the lowest-yielding feedstock at both locations, with yields being equivalent to or less than those from other feedstocks. At Becker, only fertilized anthesis yields in 'Liberty' were less than in CRP, and at Lamberton only anthesis LD + legume yields were less than in CRP. The proportions of forb and legume biomass within the CRP mix were different at each location (Figure 1-4). At Becker, forb biomass was negligible (<1%) as a result of dry conditions, and legume biomass composed less than 17% of total biomass for any N rate. Legume biomass at Becker was lower with N fertilization relative to the control. At Lamberton, forb biomass was between 28 and 48% of total yield, and forb yield at 112 kg N ha^{-1} was greater than for other N

treatments. Legumes accounted for less than 5% of total biomass regardless of fertilization treatment at Lamberton, and N fertilization did not affect legume biomass yield. In general, our results are similar to other studies that found a high-diversity polyculture yielded less than the most productive switchgrass monocultures (e.g. Mangan et al. 2011; Jungers et al. 2015; Duran et al. 2016).

Annual biomass yields

To evaluate annual response to treatment effects at each location, we split the analysis by year (Tables 1-9 and 1-10) and have selected four of the six feedstocks to discuss on an annual basis: 'Liberty' and 'Shawnee' switchgrass, the CRP mix and the LD grass mix. 'Sunburst' yields are comparable to those of 'Shawnee', and adding legumes to the LD mix either made no difference or decreased average yields at both locations. We will also discuss only the feedstock × N interaction for the post-frost harvest regime, which is likely to be the most viable harvest management practice for stand longevity at northern latitudes (Casler and Boe 2003).

Becker

In Becker, 2013 biomass yields were adversely impacted by dry conditions (Figure 1-5A), averaging 0.44 Mg DM ha^{-1}. In comparison, Mangan et al.'s (2011) first year post-establishment yield at Becker averaged 1.2 Mg DM ha^{-1} across 12 feedstocks. Maximum yield of any feedstock in 2013 was 0.93 Mg DM ha^{-1}, in fertilized 'Shawnee' switchgrass. 'Shawnee' produced more biomass than any other feedstock in 2013, suggesting greater

drought-tolerance than ‘Liberty’ or either polyculture. Yield response to N application, however, was likely limited as a result of drought conditions. Polyculture yields did not increase in response to N, and while yields in ‘Liberty’ and ‘Shawnee’ increased with N application, there were no yield differences between 56 and 112 kg N ha^{-1}.

In 2014, the average yield was 2.31 Mg DM ha^{-1} (Figure 1-5B), more than five times greater than the 2013 yield. Yields of ‘Liberty’ and ‘Shawnee’ were similar at 0 and 56 kg N ha^{-1}, despite reduced ground cover percentage in ‘Liberty’. Visual observations of ‘Liberty’ plants were consistent with published descriptions of lowland ecotypes (Porter 1966; Parrish and Fike 2005): ‘Liberty’ plants were taller, had thicker stems, and longer and wider leaves relative to upland cultivars ‘Shawnee’ or ‘Sunburst’. Fewer plants, therefore, would be necessary to produce yields comparable to ‘Shawnee’. However, ‘Shawnee’ yielded more than ‘Liberty’ at 112 kg N ha^{-1}, likely as a result of weed competition in ‘Liberty’. Yields in the LD mix were comparable to ‘Shawnee’ at all N rates, while the CRP mix produced equivalent or less biomass than all other feedstocks. Yields of grass feedstocks increased with N application, but only ‘Shawnee’ produced more biomass at 112 than at 56 kg N ha^{-1}.

Average yield in 2015 was 3.52 Mg DM ha^{-1} (Figure 1-5C), 50% greater than in 2014, and eight times greater than in 2013, reflecting both adequate precipitation and increased stand maturity. As in 2014, ‘Shawnee’ and ‘Liberty’ were similar at 56 kg N ha^{-1}, but ‘Shawnee’ produced more at 112 kg N ha^{-1}. Contrary to 2014 yield results, there was no difference between ‘Liberty’ at 56 and 112 kg N ha^{-1}, suggesting that ‘Liberty’ was recovering from earlier setbacks in stand productivity. Similar to 2014, the LD mix

produced as much as 'Shawnee' at 0 and 112 kg N ha^{-1}, although LD mix yield was less than 'Shawnee' at 56 kg N ha^{-1}. All CRP mix yields were less than or equivalent to unfertilized yields in all other feedstocks, similar to 2013 and 2014, suggesting the CRP mix may not be well-adapted to extremely dry, nutrient-poor sites.

According to Stout and Jung (1995), yield response to increasing N application would be expected on soils with low fertility, similar to those at Becker. Our feedstocks demonstrated a mixed response that varied by year, with only some showing greater growth at an increased N application rate. Nitrogen acquisition in coarse-textured soils may depend on several factors such as precipitation frequency and intensity as well as stand maturity and associated rooting depth. For example, in 2013 and 2014, yields of the LD mix did not increase at 112 kg N ha^{-1} relative to 56 kg N ha^{-1}, but did increase in response to increased N application in 2015, and yields of 'Shawnee' were different between 56 and 112 kg N ha^{-1} only in 2014. Variability in precipitation patterns and/or stand maturity could account for these annual differences.

Lamberton

At Lamberton, average yield in 2014 was 7.2 Mg DM ha^{-1} (Figure 1-6A). Maximum productivity occurred in 'Shawnee' at all levels of N as well as 'Liberty' and LD mix fertilized at 112 Mg N ha^{-1}. There was no yield response to N in any feedstock other than the CRP mix, which only produced greater biomass at 112 kg N ha^{-1} relative to the control, likely as a result of forb biomass response to N. Overall, the muted response to N was likely a result of N mineralization from organic matter.

In 2015, average yield was 8.7 Mg DM ha^{-1} (Figure 1-6B) and the increase in biomass between 2014 and 2015 resulted largely from yield gains in in the polycultures. Average LD mix yield increased from 7.1 to 12.7 Mg DM ha^{-1} yr^{-1} and average CRP mix yield increased from 5.1 to 7.1 Mg DM ha^{-1} yr^{-1}. Fertilized LD mix was the most productive feedstock in 2015, averaging 13.9 Mg DM ha^{-1}. Fertilized 'Shawnee' averaged 10.39 Mg DM ha^{-1}, and 'Liberty' yields were less than both LD and 'Shawnee' but equivalent to CRP for all N treatments. No feedstock exhibited yield differences between 56 and 112 kg N ha^{-1} in 2015. Overall, N response in 2015 was more pronounced than in 2014: yields of CRP mix, LD mix and 'Shawnee' were significantly greater at 56 kg N ha^{-1} and 'Liberty' was significantly greater at 112 kg N ha^{-1} relative to the control.

Average yield in 2016 was 10.2 Mg DM ha^{-1} (Figure 1-6C), with year-over-year biomass increases in all feedstocks except the LD mix. The most productive feedstocks in 2016 were 'Shawnee' and the LD mix, fertilized at 56 and 112 kg N ha^{-1}, as well as the CRP mix fertilized at 112 kg N ha^{-1}. Similar to 2014 and 2015, 'Liberty' produced less biomass than LD and 'Shawnee' but was similar to CRP for all N treatments. 'Liberty', CRP, and LD all exhibited greater yield at 112 kg N ha^{-1} relative to the control, but there were no differences in 'Shawnee' for any N treatment. CRP was the only feedstock exhibiting increased yield at 112 kg N ha^{-1} relative to 56 kg N ha^{-1}.

Similar to annual results at Becker, 'Shawnee' and the LD mix were the most productive feedstocks, and all feedstocks differed in their response to N fertilization in each year. However, most feedstocks did not exhibit increased yield at 112 kg N ha^{-1}

relative to 56 kg N ha^{-1}, suggesting an optimum N application rate of 56 kg N ha^{-1} at Lamberton.

Nitrogen concentration, removal, and use efficiency

At both locations, there were harvests treatment interaction effects on average N tissue concentration and total N removal. At Becker, the harvest × feedstock × N rate interaction was significant for both N tissue concentration and N removal ($p < 0.001$ for both), and at Lamberton the harvest × N rate interaction was significant for both tissue N concentration and N removal ($p < 0.001$ for both). These interactions were largely a result of N translocation from aboveground to belowground tissue during senescence in perennial warm-season grasses (Vogel et al. 2002), but not in forb or legume biomass. N tissue concentration and N removal in grass-only plots, therefore, was less in the post-frost relative to the anthesis harvest, but not in plots containing legumes and forbs. Therefore, we decided to analyze N concentration and removal separately for each harvest treatment.

Nitrogen concentration

There was a feedstock × N rate interaction effect on N concentration in the anthesis harvest at Becker ($p < 0.001$) and in the post-anthesis harvest at Lamberton ($p = 0.005$). Because the interactions arose as a result of magnitude differences among tissue N concentration in unfertilized feedstocks containing legumes relative to grass-only feedstocks, we will focus our discussion on main effects to simplify the interpretation of results. At both locations, increased N application rates led to increased N tissue

concentration in both harvests (Table 1-11A), which is consistent with numerous other studies (e.g. Vogel et al. 2002; Mulkey et al. 2006; Guretzky et al. 2011; Waramit et al. 2011). In the post-frost harvest at Lamberton, N tissue concentration was similar for both the control and 56 kg N ha^{-1} treatments, largely as a result of legume biomass. Inclusion of legumes in grass mixes can increase biomass N concentration (Posler et al. 1993), and the proportion of leguminous biomass may account for this difference. Legumes in CRP and LD + legumes feedstocks in the post-frost harvest at Lamberton comprised an average of 9.2% biomass by wet weight, whereas at Becker they comprised only 3.8%, likely not enough to increase N concentration in unfertilized treatments.

N concentration in feedstocks containing legumes was equal to or greater than grass-only feedstocks at both sites (Table 1-11B). The grass-only feedstocks were equivalent in tissue N concentration in the anthesis harvest at both locations, but exhibited differences in the post-frost harvest. At Becker, N tissue concentration in 'Sunburst' was less than other feedstocks in the post-frost harvest. In contrast, at Lamberton, N concentration in 'Sunburst' was similar to all but 'Liberty', which had lower N concentration than both 'Sunburst' and LD. Variation in nitrogen dynamics among warm-season grasses and even among switchgrass cultivars is not surprising. However, drawing conclusions about nitrogen dynamics among feedstocks can be complicated by factors such as N timing, variation between studies and annual variability in climate (Brejda 2000).

Nitrogen removal

At Becker, there were feedstock × nitrogen interaction effects ($p < 0.001$) for both harvest treatments, arising from differences in N uptake among feedstocks with and without legumes and variability in yield among grass-only feedstocks (Figure 1-7). Nitrogen removal generally increased with increasing N application in grass-only feedstocks for both harvest treatments. N removal in the LD mix was similar between 56 and 112 kg N ha^{-1} in the near-anthesis harvest as a result of similar yields, and N removal in 'Liberty' was similar between 56 and 112 kg N ha^{-1} in the post-frost harvest as a result of yield reduction arising from weed pressure at 112 kg N ha^{-1}. Nitrogen removal in the CRP mix was similar under fertilized conditions for both harvest treatments, reflecting patterns in yield. In the LD + Legume feedstock, N removal was similar between the control and 56 kg ha^{-1} N in the anthesis harvest, reflecting greater N concentration in legume biomass (34.6% of total wet weight biomass) under unfertilized conditions. In the post-frost harvest, N removal reflected patterns in yield rather than in N concentration; legume biomass accounted for only 6.6% of wet weight biomass.

At Lamberton, there was a feedstock × nitrogen interaction effect ($p = 0.026$) for the anthesis harvest. Similar to N concentration, however, the interaction was a result of differences in tissue N concentration magnitude among unfertilized feedstocks containing legumes relative to grass-only feedstocks; we will therefore focus only on main effects to simplify the interpretation of results. There was no feedstock × nitrogen interaction for the post-frost harvest at Lamberton ($p = 0.090$). Nitrogen removal increased with increasing N application at both sites (Table 1-12) as a function of increased N concentration and

increased biomass yields. Average N removal for the anthesis and post-frost harvest was 167.8 and 88.3 kg N, respectively. Nitrogen removal relative to the control increased by approximately 35 and 80% at 56 and 112 kg N ha^{-1}, respectively, for both harvest treatments. The polycultures at Lamberton removed as much if not more N than the most productive switchgrass cultivars, largely as a result of annual legume and forb N uptake. 'Liberty' was among the lowest in N removal under both harvest regimes, largely as a result of lesser biomass production relative to other grass-only feedstocks. In general, N removal reflected biomass production, but annual legumes and forbs increased N removal relative to grass-only feedstocks when they produced sufficient biomass.

Nitrogen removal under unfertilized conditions at Lamberton was several times greater than unfertilized N removal at Becker for each harvest, reflecting greater soil fertility and reduced need for fertilizer N (Stout and Jung 1995) at Lamberton. Because N removal is largely reflective of yield, variability in N removal among feedstocks is not unexpected, given yield differences that arise as a result of cultivar x environment interactions (Casler and Boe 2003). As anticipated, N removal in the post-frost harvest was generally less than in the near-anthesis harvest, reflecting translocation of N following senescence. In the anthesis harvest, N removal at Lamberton exceeded applied N only for 56 kg N ha^{-1} yr^{-1}, suggesting that additional N was mineralized from soil, similar to results from Lemus et al. (2009). In all other near-anthesis fertilized treatments at both locations, N removal did not exceed applied N, suggesting that N was either used for increasing belowground biomass (Jungers et al. 2015b) or lost to the environment.

Fertilizer nitrogen use efficiency

At Becker, there was a harvest × feedstock × N rate interaction effect on NUE ($p < 0.001$), largely as a result of differences in magnitude between the anthesis and post-frost harvests in LD + legumes and 'Liberty' (Figure 1-8). For all feedstocks at Becker, NUE averaged 11.9 and 21.0% in the anthesis and post-frost harvests, respectively. This difference reflects greater yields with N fertilization in the post-frost relative to the anthesis harvest. All feedstocks had equal or lesser NUE with increased N application at Becker, which was expected given that increases in N application have been shown to result in diminished NUE (Brejda 2000), likely as a result of limitations in other resources, including moisture (Jungers et al. 2015b).

At Lamberton, feedstock was the only treatment difference in NUE ($p = 0.004$) (Table 1-13). The lack of N rate effect on NUE is not unexpected, given that the loam soil at Lamberton has greater fertility and water-holding capacity than the soil at Becker. Jungers et al. (2015b) also found that NUE did not change with N fertilization in switchgrass at Lamberton. NUE in the LD + legume feedstock was 2.8%, less than all other feedstocks except 'Sunburst'. This result was not unexpected, given that there was no yield response to N fertilization in the LD + legume feedstock or in 'Sunburst' fertilized at 56 kg N ha^{-1} relative to the control treatment.

In a summary of 215 site-year-species-N rate combinations in warm-season native grasses, Brejda (2000) found an average NUE of 29 kg DM kg^{-1} N ha^{-1} (SD = 15) averaged across N application rates. Averaging over grass-only feedstocks, harvest

treatment and N rates, our NUE was 17.4 kg DM kg^{-1} N ha^{-1} (SD = 10.8), within the range reported by Brejda (2000).

Conclusions

Our results indicate that highly-productive, well-adapted switchgrass cultivars 'Shawnee' and 'Sunburst', as well as a low-diversity grass mix, generally produced more biomass than the bioenergy cultivar 'Liberty' switchgrass, a low-diversity grass mix plus legumes, and a 14-species high-diversity polyculture on marginal soils in Minnesota over three treatment years. We also found that 'Liberty' was susceptible to stand loss from winterkill on a drought-prone site, and it did not compete as well with weeds as did 'Shawnee' and 'Sunburst'. Both switchgrass monocultures and native prairie perennial polycultures exhibited an inconsistent response to N fertilization in this study, similar to what others have found (Parrish and Fike 2005), particularly on marginal soils.

Based on these results, best management practices for perennial biomass production on marginal soils in Minnesota should include planting either a well-adapted switchgrass variety, such as 'Sunburst' or 'Shawnee', or a low-diversity grass mix, and fertilizing with 56 kg N ha^{-1} in post-establishment years on marginal loam soils and up to 112 kg N ha^{-1} on coarser-textured soils. Producers may also have flexibility in harvest timing (anthesis or post-frost) in the first few years following establishment, but may depend on the interaction of site × cultivar. While 'Sunburst' produced similar yields under both harvest regimes at Becker, 'Shawnee' and the LD mix produced similar yields under both harvest regimes at Lamberton. However, the anthesis biomass yields and

percent ground cover could change in more advanced production years. Producers will also remove less N from the system in a post-frost regime, which may promote stand longevity with fewer inputs over time, leading to more sustainable production of biomass with native perennial grasses.

Table 1-1. Normal (1981-2010) monthly precipitation, temperature, and summary of conditions for duration of study at Becker (2012-2015) and Lamberton (2013-2016), MN. Est. = Establishment year. 'Sum' refers to annual total precipitation; 'mean' refers to mean annual temperature. Source: Midwestern Regional Climate Center, cli-MATE (MRCC Application Tools Environment, http://mrcc.isws.illinois.edu/CLIMATE/, accessed 4/2016).

Location	Month	30-year average	Year				30-year average	Year			
			Est.	1	2	3		Est.	1	2	3
		mm					°C				
Becker	January	17	14	11	34	6	-11.2	-6.6	-10.4	-15.2	-8.6
	February	17	31	34	30	9	-8.2	-4.1	-9.6	-14.7	-12.9
	March	37	29	67	31	10	-1.3	6.6	-5.6	-5.3	0.2
	April	65	66	74	150	42	7.0	8.7	2.3	4.9	8.0
	May	81	223	126	171	153	13.5	15.7	12.4	14.2	12.8
	June	99	60	146	157	118	18.6	20.4	18.4	20.2	19.0
	July	87	91	36	32	182	21.1	24.7	21.6	21.2	21.5
	August	98	31	22	142	79	19.8	19.9	20.9	21.8	19.5
	September	89	6	47	103	57	14.7	15.3	17.2	16.2	18.2
	October	59	19	110	17	80	7.5	6.9	7.2	9.0	9.2
	November	34	27	14	48	79	-1.4	0.5	-1.8	-5.1	3.4
	December	22	38	45	19	26	-8.7	-7.8	-14.3	-4.4	-3.0
	Sum/mean	704	636	733	933	841	5.9	8.4	4.9	5.2	7.3
Lamberton	January	16	7	18	11	8	-9.6	-8.7	-12.7	-7.3	-9.2
	February	16	16	13	5	18	-6.9	-8.9	-12.6	-11.7	-4.4
	March	39	24	25	10	51	-0.5	-4.4	-4.1	1.1	3.7
	April	73	82	87	31	85	7.5	2.1	5.6	8.6	8.6
	May	86	106	46	139	141	14.6	12.8	13.8	13.9	14.7
	June	103	134	188	128	66	20.1	19.8	20.1	20.3	21.4
	July	99	9	30	96	176	22.1	22.4	20.5	21.7	22.1
	August	92	46	94	113	135	20.5	21.1	21.0	19.7	21.4
	September	86	48	154	87	134	15.8	18.5	16.3	19.5	17.7
	October	52	82	12	41	72	8.6	8.8	9.6	10.3	10.1
	November	31	12	13	84	47	-0.3	-0.9	-4.5	4.1	5.4
	December	20	22	25	34	29	-7.5	-11.9	-4.8	-2.8	-8.2
	Sum/mean	714	590	705	780	960	7.0	5.9	5.7	8.1	8.6

Table 1-2. Species composition of polyculture feedstocks evaluated at Becker and Lamberton, MN, as a function of nitrogen rate and harvest treatment. Monoculture feedstocks (not shown in table) were 'Shawnee', 'Sunburst', and 'Liberty' switchgrass (*Panicum virgatum* L.).

Feedstock	Species composition
Low diversity grass mix (LD)	'Bonanza' and 'Goldmine' big bluestem, *Andropogon gerardii* Vitman 'Scout' and 'Warrior' indiangrass, *Sorghastrum nutans* L. 'Butte' sideoats grama, *Bouteloua curtipendula* Michx.
Low diversity grass mix plus legumes (LD + legumes)	'Bonanza' and 'Goldmine' big bluestem, *Andropogon gerardii* Vitman 'Scout' and 'Warrior' indiangrass, *Sorghastrum nutans* L. 'Butte' sideoats grama, *Bouteloua curtipendula* Michx. Purple prairie clover, *Dalea purpurea* Vent. Canada milkvetch, *Astragalus canadensis* L. Showy tick trefoil, *Desmodium canadense* L.
High diversity polyculture (CRP)	Minnesota native big bluestem, *Andropogon gerardii* Vitman 'Rodan' western wheatgrass, *Pascopyrum smithi* (Rydb.) A. Löve 'Mandan' Canada wildrye, *Elymus canadensis* L. 'Pierre' sideoats grama, *Bouteloua curtipendula* Michx. Wisconsin native indiangrass, *Sorghastrum nutans* L. Minnesota native purple prairie clover, *Dalea purpurea* Vent. Minnesota native yellow coneflower, *Ratibida pinnata* (Vent.) Barnhart 'Lodorm' green needlegrass, *Nassella viridula* (Trin.) Barkworth 'Sunburst' switchgrass, *Panicum virgatum* L. Bad River Ecotype blue grama, *Bouteloua gracilis* (Willd. Ex Kunth.) Lag. Ex Griffiths Iowa native black-eyed Susan, *Rudbeckia hirta* L. Hoary vervain, *Verbena stricta* Vent. Wild bergamot, *Monarda fistulosa* L. Minnesota native white prairie clover, *Dalea candida* Michx.

Table 1-3. Establishment-year irrigation at Becker, MN.

Date	Irrigation
	mm
22-Jun	10
25-Jun	13
27-Jun	8
28-Jun	13
2-Jul	15
5-Jul	15
9-Jul	15
12-Jul	13
16-Jul	15
19-Jul	6
23-Jul	13
26-Jul	10

Table 1-4. Herbicide and mechanical weed control methods, rates, and dates of application in grass-only plots at Becker and Lamberton, MN.

	Becker		Lamberton	
Year	Date	Treatment	Date	Treatment
2012	None		N/A	
2013	25-Jun	Tall mowing to remove weed seed heads	20-Jun	2.34 L ha^{-1} atrazine (2-chloro-4-ethylamino-6-isoproplyamino-s-triazine) on switchgrass, 0.29 L ha^{-1} imazapic (5-methyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid) on LD mix
	23-Jul	2.34 L ha^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid)		
2014	13-Jun	2.34 L ha^{-1} 2,4-D	27-Jun	2.34 L ha^{-1} 2,4-D
	30-Jun to 3-Jul	Hand weeding		
	10-Jul	2.34 L ha^{-1} 2,4-D		
	14-Oct	2.34 L ha^{-1} 2,4-D		
2015	15-Apr	2.34 L ha^{-1} 2,4-D	4-Jun	2.34 L ha^{-1} 2,4-D
	13-May	0.18 kg ha^{-1} Opensight™ (62% Aminopyralid Potassium, 9% Metsulfuron-methyl)		
2016	22-Mar	2.69 kg ha^{-1} mesotrione (2-(4-methylsulfonyl-2-nitrobenzoyl)cyclohexane-1,3-dione) with 3.17 kg ha^{-1} glyphosate (N-(phosphonomethyl)glycine)	18-May	2.34 L ha^{-1} 2,4-D

Table 1-5. Fixed-effect results from analysis of variance on biomass, summed over three treatment years, at Becker and Lamberton, MN.

Source of variation	Num DF	Den DF	Becker		Lamberton	
			F statistic	*P* value	*F* statistic	*P* value
Harvest (H)	1	3	11.2	0.044	8.1	0.066
Feedstock (F)	5	30	28.1	<0.001	22.4	<0.001
Nitrogen (N)	2	72	203.2	<0.001	52.2	<0.001
H x F	5	30	10.4	<0.001	5.5	0.001
H x N	2	72	14.7	<0.001	0.7	0.518
F x N	10	72	7.6	<0.001	2.1	0.031
H x F x N	10	72	5.0	<0.001	0.9	0.567

Table 1-6. Year × grass interaction effects on grass percent ground cover for treatment years 1 and 2 at Becker (2013-2014) and Lamberton (2014-2015), MN. Within columns at each site, values sharing the same letter are not significantly different ($p \leq 0.05$). Least significant difference between years (across rows) is 7.93 at Becker and 2.93 at Lamberton, ns = no significant differences.

		1	2
		%	
Becker	LD	59.3 c	67.3 b
	LD + legumes	64.4 c	61.1 b
	Liberty	78.6 b	45.5 c
	Shawnee	94.1 a	83.4 a
	Sunburst	96.1 a	96.2 a
Lamberton	LD	93.9 b	96.4
	LD + legumes	84.9 c	97.7
	Liberty	97.3 ab	98.8 ns
	Shawnee	98.1 ab	99.3
	Sunburst	99.6 a	99.3

Table 1-7. Harvest × feedstock interaction effect on wet weed biomass at Lamberton, MN, averaged over year. Within columns, values sharing the same letter are not significantly different ($p \leq 0.05$). Least significant difference between harvests (across rows) is 5.31.

	Near-anthesis	Post-frost
	%	
LD	9.8 b	3.7 b
Liberty	15.1 a	10.5 a
Shawnee	5.8 b	0.3 b
Sunburst	14.6 a	1.1 b

Table 1-8. Percentage of legume biomass in LD + legume feedstock summed over treatment years at Becker and Lamberton, MN. The harvest × nitrogen interaction was significant at Becker, and the harvest main effect was significant at Lamberton. Values within site sharing letters are not significantly different ($p \leq 0.05$).

	N	Near-anthesis	Post-frost
	kg ha^{-1}	%	
Becker	0	34.6 a	6.6 b
	56	7.5 bc	1.2 c
	112	8.5 bc	2.3 bc
Lamberton		22.0 a	10.4 b

Table 1-9. Annual fixed-effect results from biomass yield analysis of variance at Becker, MN.

Source of variation	Num DF	Den DF	2013		2014		2015	
			F statistic	*P* value	*F* statistic	*P* value	*F* statistic	*P* value
Harvest (H)	1	3	3.1	0.177	23.8	0.017	5.8	0.095
Feedstock (F)	5	30	29.5	<0.001	14.9	<0.001	23.0	<0.001
Nitrogen (N)	2	72	15.9	<0.001	65.3	<0.001	131.7	<0.001
H x F	5	30	5.6	<0.001	7.1	<0.001	10.9	<0.001
H x N	2	72	6.7	0.002	9.4	<0.001	6.7	0.002
F x N	10	72	5.5	<0.001	4.8	<0.001	3.9	<0.001
H x F x N	10	72	2.0	0.045	3.2	0.002	2.7	0.008

Table 1-10. Annual fixed-effect results from biomass yield analysis of variance at Lamberton, MN.

Source of variation	Num DF	Den DF	2014		2015		2016	
			F statistic	*P* value	*F* statistic	*P* value	*F* statistic	*P* value
Harvest (H)	1	3	11.4	0.043	6.5	0.084	16.9	0.026
Feedstock (F)	5	30	8.4	<0.001	42.6	<0.001	21.6	<0.001
Nitrogen (N)	2	72	18.5	<0.001	74.7	<0.001	12.2	<0.001
H x F	5	30	2.1	0.089	12.8	<0.001	6.1	<0.001
H x N	2	72	0.9	0.411	1.2	0.303	0.2	0.823
F x N	10	72	1.4	0.194	4.3	<0.001	0.6	0.845
H x F x N	10	72	0.9	0.538	0.9	0.525	1.1	0.400

Table 1-11. Nitrogen rate main effect (A) and feedstock main effect (B) on average N concentration for anthesis and post-frost harvest treatments at Becker and Lamberton, MN. Values sharing letters within harvest treatments are similar ($p \leq 0.05$).

		Becker		Lamberton	
A		Anthesis	Post-frost	Anthesis	Post-frost
		N concentration			
		%			
	0	0.95 c	0.59 c	0.82 c	0.50 b
	56	1.09 b	0.71 b	0.97 b	0.53 b
	112	1.38 a	0.92 a	1.16 a	0.67 a
B	CRP	1.2	0.91 a	1.10 b	0.73 a
	LD + legumes	1.21	0.79 b	1.29 a	0.79 a
	LD mix	1.1	0.74 b	0.93 c	0.50 b
	Liberty	1.19 ns	0.74 b	0.83 c	0.36 c
	Shawnee	1.1	0.70 b	0.86 c	0.50 bc
	Sunburst	1.0	0.58 c	0.89 c	0.51 b

Table 1-12. Nitrogen rate main effect (A) and feedstock main effect (B) on total N removal over three years for anthesis and post-frost harvest treatments at Lamberton, MN. Values sharing letters within harvest treatments are similar ($p \leq 0.05$).

		Anthesis	Post-frost
		N removal	
		kg ha^{-1}	
A	0	175.9 c	108.5 c
	56	240.5 b	143.6 b
	112	318.1 a	193.5 a
B	CRP	57.0 bc	158.1 b
	LD + legumes	69.2 ab	191.7 a
	LD mix	55.4 c	159.6 b
	Liberty	36.0 d	80.1 c
	Shawnee	80.2 a	153.9 b
	Sunburst	78.0 a	147.9 b

Table 1-13. Feedstock main effect on nitrogen use efficiency averaged over treatment years at Lamberton, MN. Treatments sharing letters are similar ($p \leq 0.05$).

Feedstock	NUE
	kg kg^{-1}
CRP	28.6 a
LD +Legumes	2.8 c
LD Mix	25.7 ab
Liberty	22.8 ab
Shawnee	35.0 a
Sunburst	10.6 bc

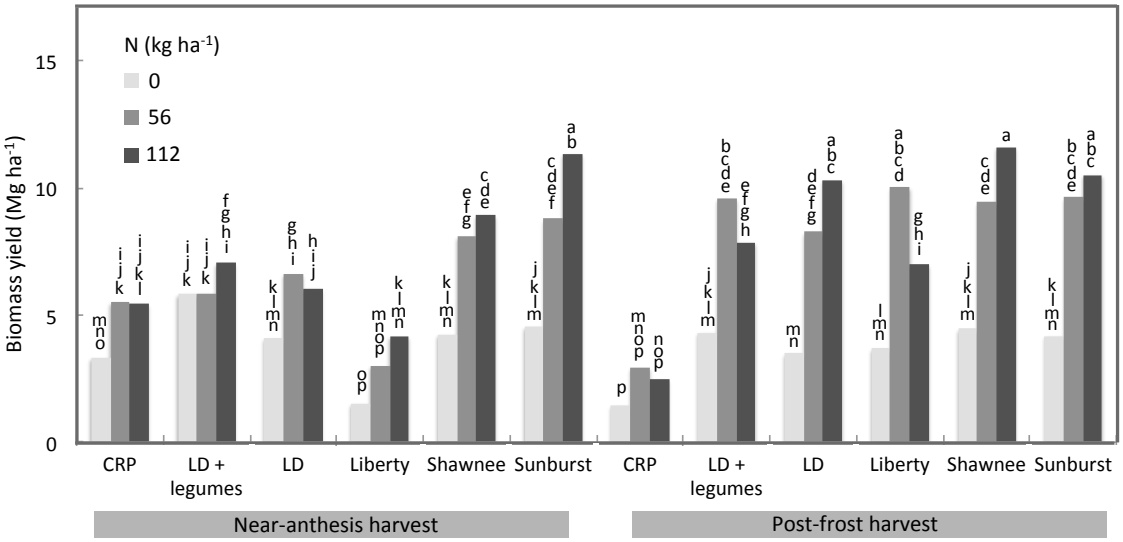


Figure 1-1. Harvest × feedstock × nitrogen interaction for total biomass yield of native perennial grasses and polycultures, summed over three treatment years at Becker, MN. Bars sharing the same letter indicate similar yield ($p \leq 0.05$). CRP = Conservation Reserve Program mix, LD = low diversity grass mix.

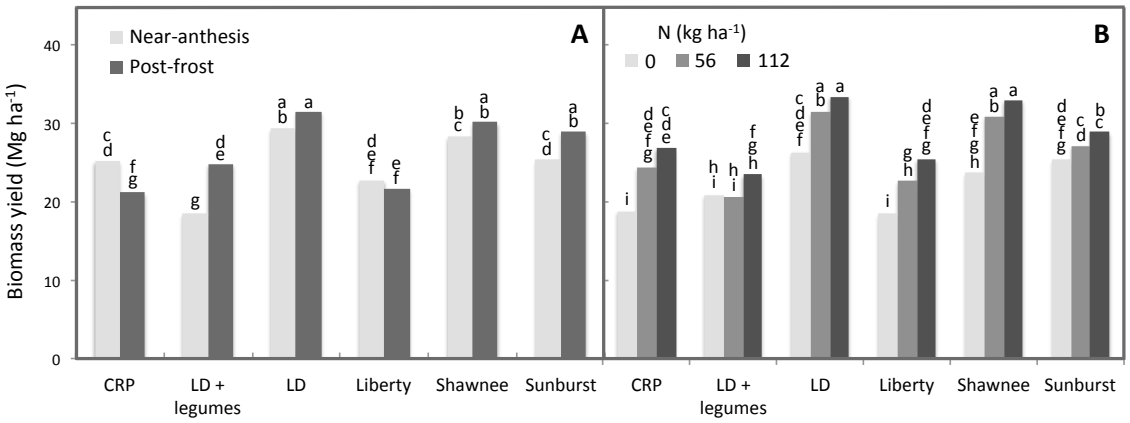


Figure 1-2. Harvest × feedstock (A) and feedstock × nitrogen (B) interaction for total biomass yields of native perennial grasses and polycultures, summed over three treatment years at Lamberton, MN. Bars sharing the same letter indicate similar yield ($p \leq 0.05$). CRP = Conservation Reserve Program mix, LD = low diversity grass mix.

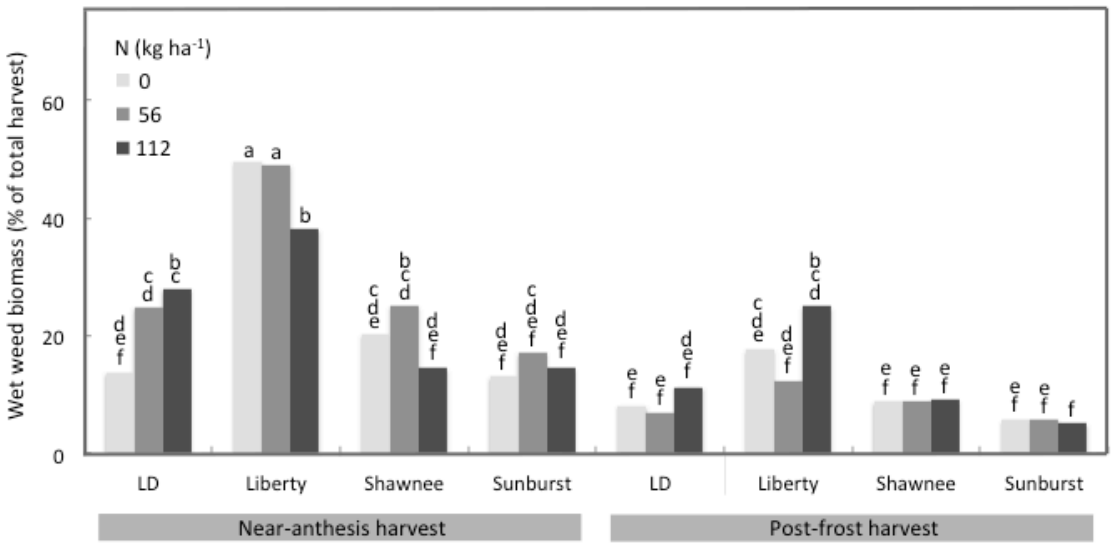


Figure 1-3. Harvest × feedstock × nitrogen interaction for wet weed biomass, as percent of total wet weight harvest, averaged over treatment years in grass-only plots at Becker, MN. Treatments sharing the same letter are similar ($p \leq 0.05$).

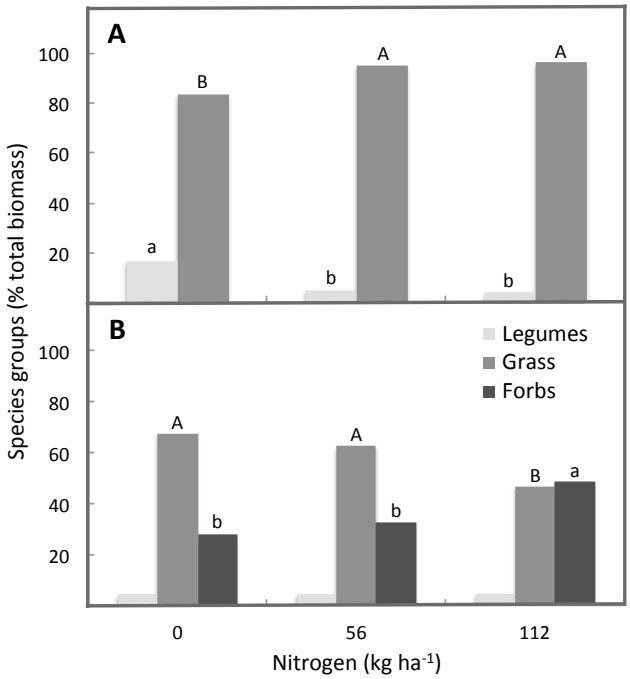


Figure 1-4. Nitrogen rate effect on percentage of species group in total CRP mix biomass summed over treatment years at A) Becker and B) Lamberton, MN. Bars sharing letters are not significantly different ($p \leq 0.05$), capital letters indicate differences between grass biomass at each site, lower case letters indicate differences between legumes at Becker and forbs at Lamberton. There were no differences between legumes at Lamberton.

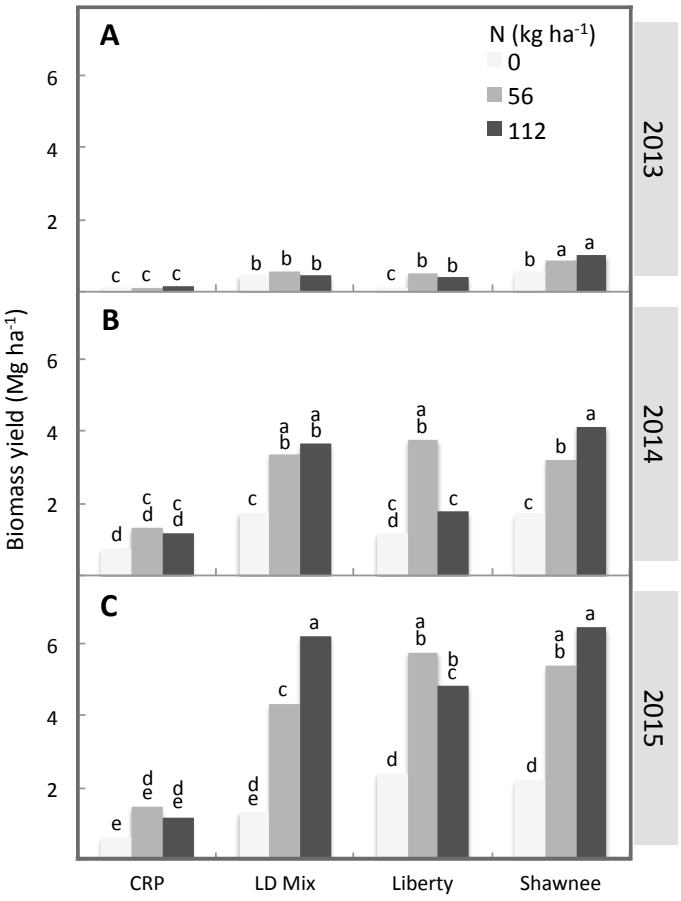


Figure 1-5. Feedstock × nitrogen rate interaction effects on post-frost biomass yield of select unfertilized and fertilized native perennial grasses and polycultures for three treatment years at Becker, MN. Bars sharing letters within years are similar ($p \leq 0.05$). CRP = Conservation Reserve Program mix, LD = low diversity grass mix.

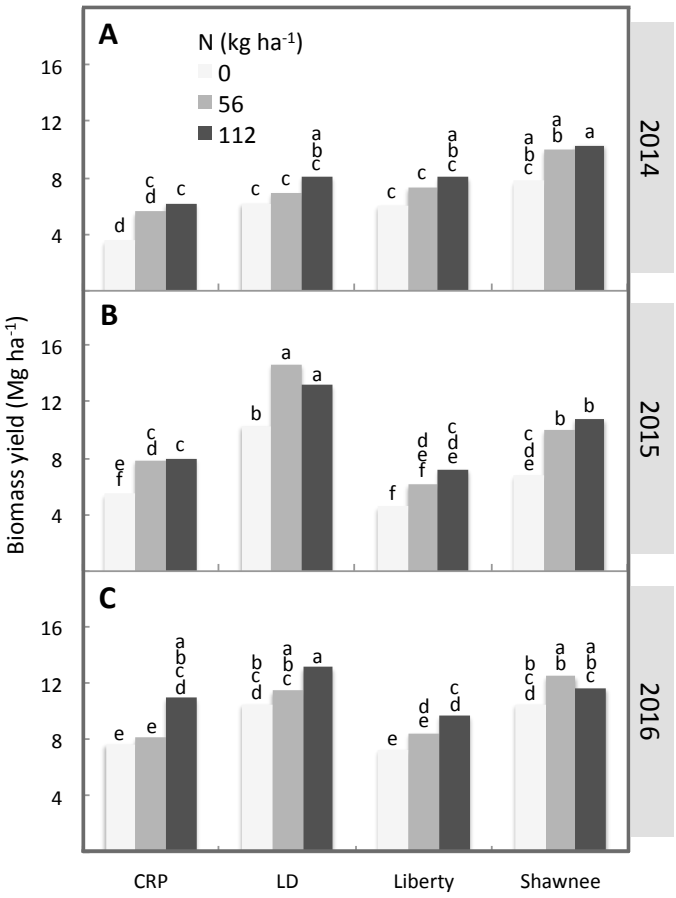


Figure 1-6. Feedstock × nitrogen rate interaction effects on post-frost biomass yield of select unfertilized and fertilized native perennial grasses and polycultures for two treatment years at Lamberton, MN. Bars sharing letters within years are similar ($p \leq 0.05$). CRP = Conservation Reserve Program mix, LD = low diversity grass mix.

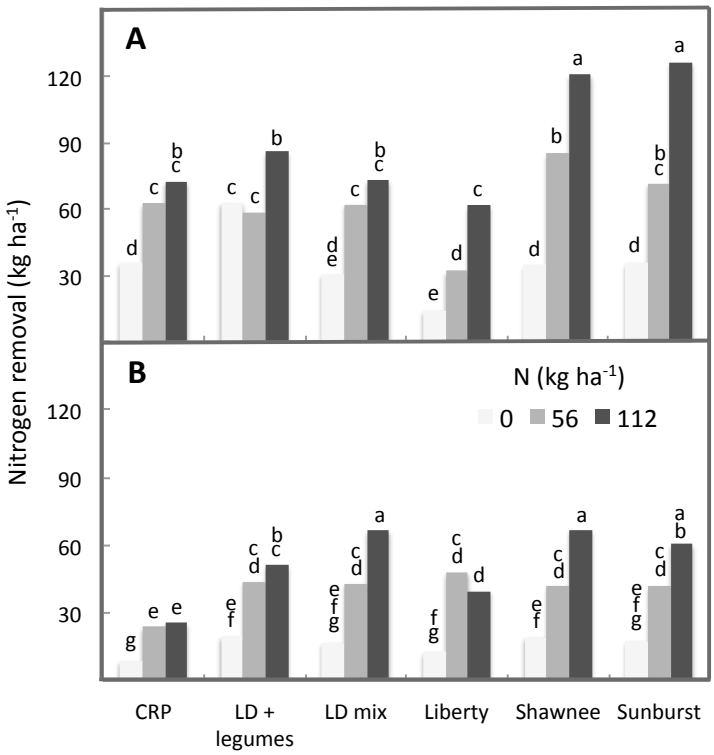


Figure 1-7. Feedstock × nitrogen rate interaction effects on total nitrogen removal over three years in unfertilized and fertilized native perennial grasses and polycultures in A) the anthesis harvest and B) the post-frost harvest at Becker, MN. Bars sharing letters within years are similar ($p \leq 0.05$). CRP = Conservation Reserve Program mix, LD = low diversity grass mix.

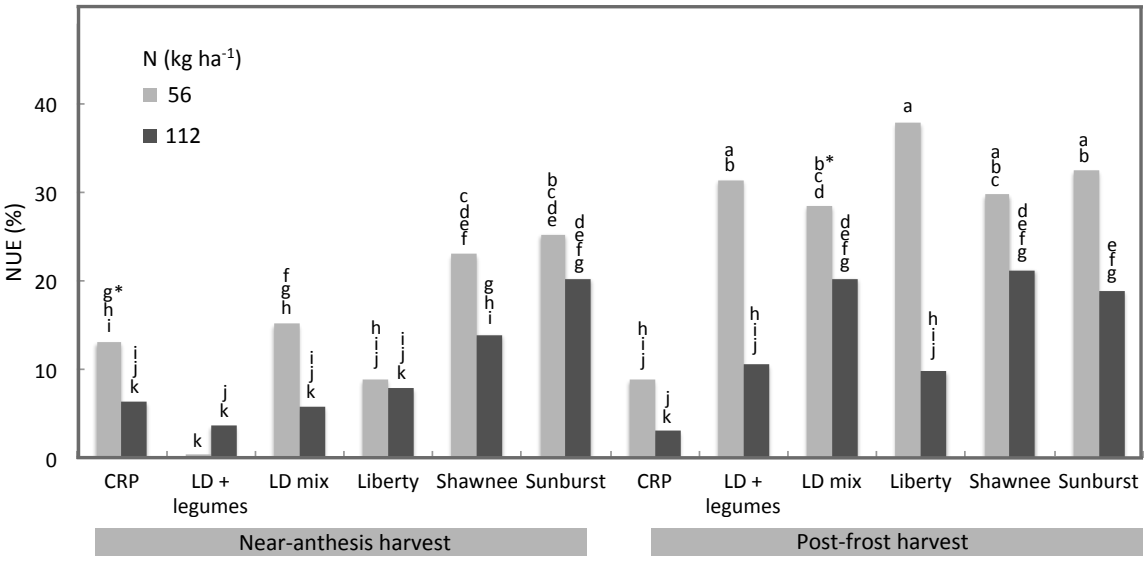


Figure 1-8. Harvest × feedstock × nitrogen interaction effects on nitrogen use efficiency of native perennial grasses and polycultures, averaged over three treatment years at Becker, MN. Bars sharing the same letter indicate similar NUE ($p \leq 0.05$). CRP = Conservation Reserve Program mix, LD = low diversity grass mix. * = Significant difference exists within feedstock pair (between N=56 and N=112) but not reflected in lettering scheme as a result of large variance.

Chapter 2 – Rhizobacteria community structure as a function of cultivar and nitrogen in switchgrass grown on two marginal soils

Abstract

Switchgrass is a native perennial grass and promising biofuel crop that can be used for production on marginal agricultural lands. As such, research into the switchgrass rhizosphere microbiome has been ongoing in an effort to identify patterns in microbial communities that may be beneficial for increasing sustainability in production. In this study, we examined the effects of cultivar and nitrogen (N) fertilization on rhizosphere bacterial community structure in switchgrass grown on two marginal soils. We selected two upland forage cultivars, 'Sunburst' and 'Shawnee', as well as the first lowland bioenergy switchgrass adapted for production in USDA hardiness zones 4, 5, and 6, 'Liberty'. We found that that existing soil characteristics primarily shaped switchgrass rhizosphere communities, but both cultivar and N fertilization also influenced microbial selection in the rhizosphere. Only N fertilization resulted in consistent differences in bacterial orders across location, including orders containing genera involved in N dynamics in soil: *Nitrosomonadales* and *Rhodocyclales*. We also found that within-site spatial variability in soil properties influenced rhizosphere community structure, although differences were confined to minor taxa (< 0.1% of sequence reads). While our results provide insight into the effects of cultivar and N fertilization on the switchgrass rhizosphere bacterial community, they also indicate a need for future research addressing

the influence of existing soil characteristics, including within-site spatial variability, on development of the rhizosphere microbiome in agricultural settings.

Introduction

Marginal agricultural lands – those that are poorly suited to annual row crops as a result of climatic or edaphic limitations, or those that pose environmental risks such as erosion – can be used to produce perennial grass feedstock for biofuels, reserving prime agricultural land for food production (Varvel et al. 2008; Gelfand et al. 2013). Switchgrass (*Panicum virgatum* L.), in particular, is considered a desirable bioenergy crop based on its broad geographic range and high yield potential on marginal lands (Vogel et al. 2002; Mitchell et al. 2008). Improved understanding of rhizosphere bacterial communities in bioenergy cropping systems such as switchgrass may enhance sustainable production on marginal lands (da Jesus et al. 2010; Liang et al. 2016), particularly in areas where fertilizer application may risk water quality degradation. Rhizosphere microorganisms can enhance crop growth by performing essential biogeochemical transformations such as organic matter decomposition and nutrient mineralization, and they utilize a number of direct and indirect plant growth promotion mechanisms such as antibiosis and improved nutrient uptake (Glick 1995; Dobbelaere et al. 2003; van Loon 2007).

In the rhizosphere, plants affect changes in the microbial community by selecting for specific populations from the community of natural bacterial inoculum in the soil (Berg and Smalla 2009; Bulgarelli et al. 2012). The composition of indigenous soil microbial communities is greatly influenced by soil characteristics, particularly pH (Fierer

and Jackson 2006), and soil type can determine both bulk and rhizosphere soil bacterial communities (Girvan et al. 2003; Ulrich and Becker 2006; Fernandez et al. 2016). Soil characteristics such as texture and nutrient levels affect plant root morphology and root exudate composition and quantity, both of which greatly influence microbial selection in the rhizosphere (Philippot et al. 2013). In soils with high nutrient availability, nutrient-mobilizing root exudates (e.g. citrate, malate) are generally less than in soils with low nutrient availability, where plant nutrient deficiencies are more likely (Neumann and Romheld 2001). Even when controlling for factors such as nutrient limitations or water stress, root exudate composition can vary significantly between soil type, likely as a function of plant-microbial feedbacks in different soils (Neumann et al. 2014).

In agricultural systems, management practices such as nitrogen (N) fertilization directly alter root exudate quantity and composition (Zhu et al. 2016) and increase N availability for microbes, affecting rhizobacterial community structure, abundance, and function (Ramirez et al. 2012). N fertilization, particularly with urea and ammonium, also alters microbial communities through increases in soil acidity (Enwall et al. 2007; Geisseler and Scow 2014). While a few studies have examined switchgrass rhizosphere bacteria in field settings under either fertilized (Hargreaves et al. 2015) or unfertilized (Xia et al. 2013; Mao et al. 2013; Mao et al. 2014a) conditions, to date there have been no studies comparing bacterial communities in the switchgrass rhizosphere as a function of N fertilization.

Switchgrass occurs in two ecotypes: upland ecotypes are adapted to northern latitudes and found primarily in areas not prone to flooding, and lowland ecotypes are

adapted to floodplains and typically have poor winter survival at northern latitudes in the US, although they generally produce greater biomass than upland ecotypes (Vogel 2000; Casler et al. 2004). Recent advances in switchgrass breeding have focused on increasing geographic adaptation of lowland cultivars through winter hardiness improvements while also selecting for seedling vigor and biomass conversion characteristics (Casler and Vogel 2014). Breeding for characteristics such as yield or disease resistance can alter the natural selection of beneficial plant microbes in the rhizosphere, and domesticated plants may be less likely to benefit from rhizosphere microflora than their wild counterparts (Philippot et al. 2013; Gopal and Gupta 2016). Rhizosphere community structure and function have been found to vary with plant selection, both over time (Siciliano and Germida 1998; Germida and Siciliano 2001; Wen et al. 2017) and between contemporary cultivars (Inceoğlu et al. 2012; Knox et al. 2014; Winston et al. 2014). While one study found that rhizosphere community structure and function varied between switchgrass ecotypes and growth stages in a greenhouse study, the two cultivars were chosen based on broad differences in climate adaptation and productivity (Casler 2012; Rodrigues et al. 2016). A better understanding of how important microbe-plant interactions differ among switchgrass cultivars adapted to the same geographic region can be particularly important for sustainable production on marginal lands, where fertilization options may be limited and soil edaphic conditions are highly variable.

Our objective was to examine the effects of cultivar and N fertilization on rhizosphere bacterial community structure, at two locations in Minnesota, using Illumina amplicon sequencing of the 16S rRNA gene. Each location selected for this experiment

represented conditions under which bioenergy crop production may be economically and logistically feasible: a sloping, eroded loam with 4.9% organic matter and an excessively drained loamy sand with 1.3% organic matter.

Materials and methods

Site description and experimental design

We conducted this experiment at two University of Minnesota field stations: the Sand Plain Research Farm in Becker, MN and the Southwest Research and Outreach Center in Lamberton, MN. Location information, soil descriptions, and pre-plant soil tests are described in Table 2-1. Pre-plant soil samples from each plot were submitted to the University of Minnesota Research Analytical Laboratory for testing: Soil pH was measured in a 1:1 (v/v) soil:water solution using a Mettler Toledo Seven-Multi pH meter with an InLab Routine Pro combination electrode (Mettler-Toledo International Inc., Columbus, OH), following Watson and Brown (1998); extractable soil phosphorus (P) was measured in air-dried soil using the Bray P1 test (Frank et al. 1998) with molybdate-blue color development observed using a Brinkmann PC 900 probe colorimeter (Metrohm AG, Herisau, Switzerland); available soil potassium (K) was extracted from air-dried soil using 1 M ammonium acetate and measured using a Perkin Elmer Analyst 100 atomic emission spectrometer (PerkinElmer Inc., Waltham, MA) (Warncke and Brown 1998); and soil organic matter (SOM) was measured as a percent of dry soil following loss on ignition (Combs and Nathan 1998). Pre-plant amendments were broadcast and incorporated at Becker as follows: 67 kg ha^{-1} triple superphosphate (TSP, 0-45-0), 168 kg

ha^{-1} potassium magnesium sulfate (KMS, 0-0-22-18), and 247 kg ha^{-1} potassium chloride (KCl, 0-0-60). At Lamberton, no pre-plant amendments were applied because of the fine-textured soil and high organic matter content.

The soil at Becker is considered marginal in productivity for reasons of excessive drainage (class 4s). Previous cropping history at Becker was winter rye (*Secale cereale*) seeded annually for more than 15 years prior to study initiation. The Lamberton site is located on a 3-6% slope, which is considered marginal in productivity for erosion (class 2e). Previous crops at Lamberton were maize (*Zea mays* L.) and soybean (*Glycine max*) as part of a tillage trial conducted between 2004 and 2008 and a maize/soy rotation from 2009 to 2012.

The microbial work in this study was conducted on a portion of experimental plots established at Becker in 2012 and at Lamberton in 2013, as described in Chapter 1 (this volume). Briefly, the full experimental design was a randomized complete block, split split-plot, where main plot treatment was harvest timing (anthesis or post-frost), subplot was feedstock (three switchgrass monocultures and three native perennial polycultures), and sub-subplot was N fertilization (0, 56 and 112 kg N ha^{-1}). Nitrogen treatments were broadcast surface applied annually, post-establishment, as urea coated with urease inhibitor NBPT (N-(n-butyl) thiophosphoric triamide) to reduce volatilization losses. Plot establishment, management, harvest, post-harvest processing, and statistical analyses of biomass yield and tissue N concentration are further described in Chapter 1 (this volume).

The current study, commenced in 2014, utilized only the anthesis harvest subplots of switchgrass monocultures that were either unfertilized (control) or fertilized at 112 kg N ha^{-1}.

N was applied at Lamberton in a single application on June 3, and N application was split and applied in equal amounts at Becker, on June 2 and June 23, to minimize leaching losses. The switchgrass cultivars used in this study were 'Shawnee', 'Sunburst', and 'Liberty'. 'Shawnee' and 'Sunburst' are hardy upland forage cultivars, and 'Liberty' is the first lowland-type cultivar adapted to USDA plant hardiness zones 4, 5, and 6, bred specifically for bioenergy production (Vogel et al. 1996; Boe and Ross 1998; Vogel et al. 2014). A further description of each switchgrass cultivar can be found in Chapter 1 (this volume).

Soil sampling and laboratory analyses

At Lamberton, plots were harvested on August 14 and plant root samples were collected on August 18. At Becker, plots were harvested on August 19 and plant root samples were collected on August 20. Three individual plants in each subplot were trimmed of stubble to approximately 2 cm above ground level and removed to a depth of 15 cm using a Giddings hydraulic probe fitted with a 7 cm diameter bit and steel tube (Giddings Machine Company, Inc., Windsor, Colorado). To minimize contamination, each sample was collected into a 15 cm × 6 cm diameter plastic auger tube liner fitted with vinyl end caps (Giddings Machine Company, Inc., Windsor, Colorado). The auger bit and steel tube were cleaned with 70% ethanol between samples. Samples were stored on ice immediately after collection and transported back to the laboratory, where they were stored at 20°C while rhizosphere soil extraction was completed (approximately three weeks).

Rhizosphere soil was separated following the protocol of Fernandez et al. (2016). Individual roots were separated from plant crowns, shaken to remove non-adhering soil, and placed into sterile 50mL collection tubes. 35mL of autoclaved 0.1M $(NH_4)_2HPO_4$ with 1% gelatin buffer was added to each sample, and tubes were agitated on a horizontal shaker table for 30 minutes. Roots were removed and set aside for inclusion in biomass determination, and the soil suspension was centrifuged at 7500 × *g* for 20 minutes. After discarding the supernatant, the remaining soil pellet was stored at -80°C.

Gravimetric soil moisture was obtained by weighing approximately 12 g of field-moist soil from each soil core in a tin weigh boat and reweighing after drying at 105°C for a minimum of 24 hours. Soil chemical analyses were conducted by the University of Minnesota Research Analytical Laboratory using field-moist soil from each soil core. Bray P1 and soil pH were evaluated as described for pre-plant soil analyses. Soil NO_3-N and NH_4-N were evaluated on air-dried samples as follows: NO_3-N was extracted with 0.01 M $CaSO_4$ and NH_4-N was extracted with 2 M KCl, with the resulting filtrate from each test measured on a Lachat Quikchem 8500 Flow Injection Analyzer (Hatch Company, Loveland, CO) (Henricksen and Selmer-Olsen 1970; Keeney and Nelson 1982; Willis and Gentry 1987; Gelderman and Beagle 1998). Concentrations of soil Fe, Mn, Zn, Cu, Pb, Ni, Cd, and Cr were determined in air-dried soil by extraction using 0.005 M DTPA (diethylenetriaminepentaacetic acid), and the resulting filtrate was analyzed using an ARL (Fisons) Model 3560 inductively-coupled plasma atomic emission spectrometer (ICP-AES) (Thermo Instrument Systems Inc. (Fisons Instruments Inc. Division),

Waltham, MA) (Fassel and Kniseley 1974; Dahlquist and Knoll 1978; Baker and Amacher 1982; Whitney 1998).

Root biomass determination

Because soil core volume was consistent between samples, root biomass was used as a proxy for root density within each sample. Root biomass was determined by using a Fine Root Extraction Device (FRED) (Pallant et al. 1993), modified by Vargas et al. (personal communication, 2015), to separate soil and debris from roots. Briefly, the modified FRED consisted of a washtub with the metal bottom removed and replaced with wood, to which perforated plastic tubing was attached in a spiral pattern covering the entire bottom. A fine plastic mesh (0.4 mm) was attached to the board above the tubing. The tub was filled with water and air was forced through the plastic tubing at a pressure of 0.172 MPa, facilitating separation and floatation of fine roots via agitation with bubbles. Soil core samples were prepared the day prior to extraction: each sample was placed into a jar filled with water and stored overnight at 4°C. The loamy sand samples from Becker were separated using only the FRED. A 5% (w/v) solution of sodium hexametaphosphate was added to the loam samples from Lamberton to facilitate clay dispersion, after Marriott and Wander (2006), and each loam sample was agitated on a horizontal shaking table for 30 minutes prior to root extraction. The FRED was cleaned and filled with fresh water prior to each sample, and as samples were put into the FRED, large roots were removed by hand and swirled in the water to loosen adhering soil. Fine roots were skimmed near the surface using a 0.4 mm mesh scoop in one-minute intervals for a total of five minutes

per sample. All roots and debris that were removed from the FRED were placed into a shallow tray with water, and roots were separated from organic debris using tweezers for a total of 10 minutes per sample. Fine roots were placed into a lined paper bag for drying, and large roots were placed into the same bag after severing from the plant crown. Roots that had been previously removed from each sample for rhizosphere soil separation were added back, and all roots were dried in a 60°C oven for a minimum of 48 hours and weighed to determine biomass.

16S rDNA sequencing

DNA was extracted from each rhizosphere soil sample using MoBio PowerSoil kits (MoBio Laboratories, Carlsbad, CA). Amplicon preparation and sequencing was performed by the University of Minnesota Genomics Center (UMGC, Minneapolis, MN). The V5V6 hypervariable regions of the 16S rRNA gene were amplified using the BSF784/R1064 primer set (Claesson et al. 2010). Illumina (San Diego, CA, USA) sequencing adapters and indices were then added by UMGC using the dual index method (Gohl et al. 2016). Sterile water negative controls were carried through amplification and sequencing. Samples were paired-end sequenced on the Illumina HiSeq 2500 platform at a read length of 150 nucleotides.

Sequence data were processed using the gopher-pipelines automated analysis (Garbe 2013). Paired-end reads were trimmed for adapters with Trimmomatic (v 0.33) (Bolger et al. 2014), to a minimum length of 50 bases, and leading and trailing 3 bases were removed. Paired reads were then merged with Pandaseq (v 2.8.1) (Masella et al.

2012) and merged reads were processed with QIIME (v 1.9.1) (Caporaso et al. 2010b). Sequences were clustered using open reference operational taxonomic unit (OTU) picking with USEARCH clustering at 97% OTUs and aligned to the Greengenes database (v 13_8) (DeSantis et al. 2006; Caporaso et al. 2010a; McDonald et al. 2012). Chimeras were removed with USEARCH61 (Edgar 2010; Edgar et al. 2011). Samples were rarefied to an even depth of 83,714 sequences per sample and QIIME was used to calculate alpha and beta diversity statistics. Phylogentic trees were made using the FastTree method in QIIME (Price et al. 2010). Sequence data were deposited to the Sequence Read Archive of the National Center for Biotechnology Information under accession number PRJNA387437.

Data analysis

Yield and N concentration data were analyzed using mixed linear models (PROC MIXED) in the SAS software program, Version 9.4 of the SAS System for Windows (Copyright © 2002-2012, SAS Institute Inc., Cary, North Carolina). Analysis of root biomass and soil parameters for each sample were analyzed using one-way analysis of variance in PROC GLM in the SAS software program. Each plant and its associated soil parameters were treated as individual subsamples and were incorporated into the analysis of variance through isolation of the subsampling error within the model, thereby reducing the total experimental error variance (Steel et al. 1997, p.223). Correct fixed-effect tests for significance were specified using test statements. Soil physiochemical parameters based on field location (block) were analyzed using PROC GLM. In all analyses, replication was treated as a random effect; all other effects were treated as fixed.

Analysis of variance on richness and diversity indices were performed using PROC GLM as described for root biomass, above. QIIME's implementation of analysis of similarity (ANOSIM), run on the rarefied UniFrac beta diversity distances, was used to test treatment effects of rate and cultivar as well as location effects of block. Principal coordinate analyses (PCoA), performed in QIIME utilizing Bray-Curtis distance matrices (Bray and Curtis 1957), were used to visualize patterns in microbial community structure. Linear discriminant analysis (LDA), used to determine differences in taxa as a function of treatment, was performed using the LDA Effect Size (LEfSe) tool (Segata et al. 2011) in Galaxy (Afgan et al. 2016). To assess and visualize soil/plant physiochemical associations with bacterial orders, a redundancy analysis (RDA) was prepared in R-studio (v 1.0.34) (R Core Team 2016) utilizing the vegan package. Variance partitioning was performed using constrained RDA as described by Borcard et al. (1992). Spearman correlation values associated with the RDA were generated using XLSTAT Ecology (v 18.07) (Addinsoft 2017). PCoA graphics were prepared in R-studio (v 1.0.34) (R Core Team 2016) utilizing ggplots2 (Wickham 2009) and Wes Anderson Palettes (https://github.com/karthik/wesanderson). Unless otherwise noted, all statistical analyses were evaluated based on $\alpha = 0.05$. To minimize multiple comparison errors, a Bonferroni correction was applied to Spearman correlation values.

Results

Treatment effects on plant parameters

At Becker, switchgrass dry matter (DM) yield differed by N application rate ($p<0.001$), with fertilized yields averaging 2.6 Mg DM ha^{-1} and unfertilized yields averaging 1.0 Mg DM ha^{-1}. Yields as a function of cultivar, from 'Liberty', 'Shawnee', and 'Sunburst', averaged 1.2, 1.9, and 2.2 Mg DM ha^{-1}, respectively ($p=0.054$). At Lamberton, there were no differences in yield in response to N fertilization ($p = 0.217$) or cultivar ($p = 0.143$), averaging 10.0 ± 2.3 Mg DM ha^{-1} overall.

Plant tissue N concentration at Becker was affected by the cultivar × N fertilization interaction ($p=0.012$) (Table 2-2). The interaction was a result of a difference in N concentration magnitude in fertilized 'Sunburst' relative to fertilized 'Liberty' and 'Shawnee'. At Lamberton, plant tissue N concentration differed as a function of N fertilization main effect ($p<0.001$), but not cultivar ($p=0.411$). N concentration in unfertilized switchgrass averaged 0.7% compared to 1.1% in fertilized switchgrass.

Root biomass increased with N fertilization at Becker ($p < 0.001$), where unfertilized root biomass averaged 2.2 g and fertilized root biomass averaged 2.6 g. There were no differences in root biomass between N treatments at Lamberton ($p=0.380$), averaging 3.5 g overall. Root biomass did not differ as a function of cultivar at either location ($p=0.052$ at Becker, $p = 0.066$ and Lamberton).

Treatment effects on soil parameters

Treatment effects on soil NO_3-N were evident at both locations (Table 2-3). At Becker, there was a cultivar x N fertilization interaction effect on soil NO_3-N ($p = 0.020$), as a result of greater soil NO_3-N in samples from fertilized 'Liberty' plots (22.7 ppm) relative to soil from other plots (averaging 5.7ppm). Greater NO_3-N in soil sampled from fertilized 'Liberty' plots was likely a result of poor plant populations relative to other treatments (Chapter 1, this volume). At Lamberton, both cultivar and N fertilization main effects influenced soil NO_3-N. Soil samples from 'Liberty' plots had greater NO_3-N than samples from 'Shawnee' or 'Sunburst' plots ($p=0.026$), although the difference was much less than at Becker. Soil NO_3-N was nearly three times greater in soil sampled from fertilized treatments at Lamberton relative to unfertilized treatments ($p < 0.001$), 12.04 and 4.69 ppm, respectively.

No treatment differences were detected for soil NH_4-N at either location, indicating that NH_4^+ resulting from urea fertilizer hydrolysis had been taken up by plant roots and/or converted to NO_3^- via nitrification. Volatilization losses were likely minimal as a result of applied urease inhibitor NBPT and pre-plant soil pH less than 6.5 at both locations.

Soil pH in samples from fertilized plots was lower than in unfertilized plots at both locations ($p = 0.013$ at Becker, and $p < 0.001$ at Lamberton) as a result of nitrification following urea fertilizer application. Soil pH at Becker was 5.96 and 5.81 for unfertilized and fertilized plots, respectively, and at Lamberton, soil pH was 5.13 and 5.02 for unfertilized and fertilized plots. At Becker, extractable soil Mn was greater in fertilized

relative to unfertilized plots ($p = 0.009$), 14.7 and 13.2 ppm, respectively. No other soil parameters were affected by cultivar or N treatment.

Bacterial community composition

A total of 72 samples were analyzed at each location, with mean Good's coverage of 94.2% ± 0.5% and 95.8% ± 0.3% at Becker and Lamberton, respectively. 11,493 operational taxonomic units (OTUs) were identified at Becker and 10,328 OTUs were identified at Lamberton. Individual rhizosphere soil samples had a mean 5,304 ± 96 OTUs at Becker and 4,157 ± 72 OTUs at Lamberton. The Chao 1 richness estimate was 17,789 ± 197 at Becker and 12,734 ± 117 at Lamberton. There were no treatment effects on bacterial community richness at either location. Diversity (Shannon index) averaged 10.78 ± 0.07 at Becker and 10.15 ± 0.05 at Lamberton. Cultivar and N treatments did not affect diversity at Lamberton, but fertilized 'Liberty' at Becker had less diversity than other treatments (cultivar × N interaction, $p = 0.020$, Table 4).

OTUs were classified into 172 orders at Becker and 173 orders at Lamberton, with 5.1% of reads unclassified at the order level at both locations. The most abundant 12 orders at each location are shown in Figures 2-1 (Becker) and 2-2 (Lamberton), and are separated by cultivar and N treatment. At Becker, the most abundant bacterial orders were *Actinomycetales* (9.3, 10.0, and 9.7% in 'Liberty', 'Shawnee', and 'Sunburst', and 9.6 and 9.8% in unfertilized and fertilized treatments, respectively), *Burkholderiales* (10.9, 7.7 and 7.0% in 'Liberty', 'Shawnee', and 'Sunburst', and 7.3 and 10.2% in unfertilized and fertilized treatments, respectively), and *Chthoniobacterales* (6.7, 7.4 and 7.1% in

'Liberty', 'Shawnee', and 'Sunburst', and 7.4 and 6.7% in unfertilized and fertilized treatments, respectively). The most abundant orders at Lamberton were *Burkholderiales* (12.6, 12.9, and 12.7% in 'Liberty', 'Shawnee', and 'Sunburst', and 11.7 and 13.7% in unfertilized and fertilized treatments, respectively), *Actinomycetales* (10.5, 9.4, and 9.0% in 'Liberty', 'Shawnee', and 'Sunburst', and 9.4 and 10.1% in unfertilized and fertilized treatments, respectively), and *Acidobacteriales* (7.5, 7.8 and 7.0% in 'Liberty', 'Shawnee', and 'Sunburst', and 7.7 and 7.2% in unfertilized and fertilized treatments, respectively).

Treatment effects on bacterial community composition

Ordination of Bray-Curtis dissimilarity distances by principal coordinate analysis did not reveal clear clustering of samples by cultivar or N treatment (Figure 2-3). Lack of clear visual separation on the ordination plot is likely caused by the low percent of total variation explained by axes 1 and 2 at Becker (23.45%) and Lamberton (36.81%). ANOSIM results, however, indicated that there were differences in overall bacterial community composition at both locations. While community composition differed as a result of the interaction between cultivar and N at both locations ($p < 0.001$ at Becker, $p = 0.001$ at Lamberton), the taxonomic diversity was too great to resolve discrete patterns related to the relative abundance of orders. Therefore, we explored differences as a function of cultivar and N fertilization separately. Both cultivar ($p < 0.001$) and N fertilization ($p < 0.001$) affected community composition at Becker, and at Lamberton, community structure differed as a function of N treatment ($p = 0.002$), but not cultivar ($p = 0.083$).

Linear discriminant analysis of effect size (LEfSe) was used to determine which orders differed as a function of treatment ($p < 0.05$). Of the 62 orders that were affected by N treatment at Becker (Figure 2-4A), four were among the most abundant orders overall: *Pseudomonadales* and *Burkholderiales* increased in abundance with fertilization, and *Solirubrobacterales* and *Acidobacteria 6 iii1-15* were more abundant without fertilization. Differences between cultivar were seen in five orders: *γ-proteobacteria HTCC218* and *β-proteobacteria SC-I-84* were more abundant in the rhizosphere of 'Sunburst' (Figure 2-4B), and *Thermomicrobia AKYG1772*, *Flavobacteriales*, and *Sphingobacterales* were more abundant in the rhizosphere of 'Liberty'. Two of those orders, *Flavobacteriales* and *Sphingobacterales*, also increased in abundance with N fertilization and were among the most abundant orders overall at Becker.

Fewer differences in bacterial orders as a function of cultivar or N fertilization were observed at Lamberton relative to Becker. Seven orders increased in abundance with N and eight were more abundant without N (Figure 2-5A). Of those, only *Rhizobiales*, which increased in abundance with N, was among the most abundant orders. Even though the entire community structure did not differ as a function of cultivar, there were four orders that increased in abundance in the rhizosphere of 'Liberty' relative to the other cultivars: *Pseudomonadales*, *Streptophyta*, and unclassified orders of *α-* and *γ-proteobacteria* (Figure 2-5B). Unlike at Becker, there was no overlap between orders affected by both fertilization and cultivar at Lamberton. However, most orders at Lamberton that were different as a function of N were also similarly different at Becker (Table 2-5).

Plant and soil characteristics affecting bacterial community composition

Results from redundancy analysis (RDA), used to partition variance in community structure, indicated that the bacterial community structure was more strongly associated with plant and soil parameters than with N rate or cultivar treatments at both locations. At Becker, 56.8% of the variation in community structure, taken as relative abundances of orders, was explainable by the combination of plant/soil parameters, while only 6.3% was explainable by treatment effects of cultivar and N (Figure 2-6A). The remaining 36.9% of the variance was explainable by a combination of both treatment effects and plant/soil parameters. At Lamberton, plant/soil parameters accounted for 75.5% of the total explainable variance. Treatment effects accounted for 16.2%, and joint plant/soil and treatment effects explained 8.3% of the total variance (Figure 2-6B).

While plant/soil parameters explained most of the variance in community structure at both locations, the most influential factors differed for each site (Figure 2-7). At Becker, *Pseudomonas* was correlated with increasing soil NO_3^- and plant tissue N concentration (Table 2-6) and relative abundance increased as a function of N fertilization. Similarly, *Burkholderiales* and *Sphingobacteriales* also increased in relative abundance with N fertilization and were positively correlated with tissue N concentration and soil NO_3^-, but both were negatively correlated with pH. *Pirellulales* was positively correlated with soil P and Cr, and decreased in relative abundance with N fertilization. *Rhizobiales* was positively associated with soil Pb, as was *Chloracidobacteria RB41*, which was also positively correlated with soil Cd, but neither of those orders was affected by N fertilization. At Lamberton, most associations were different from those at Becker, with

the exception of *Burkholderiales* being negatively correlated with pH and *Chthoniobacterales* being positively correlated with Pb (Table 2-7). *Chthoniobacterales* was also positively correlated with NH_4^+ and pH at Lamberton, and negatively correlated with tissue N concentration. *Rhizobiales* was positively correlated with Ni, but was not correlated with soil NO_3^- or switchgrass tissue N, despite showing an increase in relative abundance with N fertilization. Among the most strongly influenced bacterial orders, there were no correlations with root biomass, yield, or soil moisture at either location.

Within-site location effects on community structure

The analysis of community structure using ordination of Bray-Curtis dissimilarity distances by PCoA also revealed potential clustering of samples by location within each site, as determined by experimental block (Figure 2-8). While an analysis of spatial variability in community structure was not an initial objective of this study, the discovery of potential differences as a function of within-site spatial variability warranted further exploration. ANOSIM results confirmed that community structure differed as a function of block at both locations ($p = 0.001$). At Becker, there were no differences in Shannon index by block (Table 2-8), but there were differences in Chao1 community richness ($p = 0.048$). At Lamberton, both Shannon index and Chao1 community richness differed by block ($p < 0.001$ for both).

Differences in bacterial orders by block based on linear discriminant analysis are shown in Figure 2-9, although fewer orders differed in abundance as a function of block than as a function of cultivar or fertilization treatment at either location. At Becker, block

2 was enriched in *Rubrobacterales*, and at Lamberton block 4 had greater abundances of *Armatimonadales* and *Thermogemmatisporales*, and block 3 was enriched in bacteria unclassified at the order level. However, the differences in classified orders were confined to minor taxa, < 0.1% of sequence reads.

Soil physiochemical parameters were also found to vary as a function of block at each site (Table 2-9). At Becker, all parameters other than soil NO_3^- differed by block, with soil moisture, NH_4^+, Fe, Mn, Zn, Cu, Pb, Ni and Cd all greater in block 4 relative to other blocks. At Lamberton, soil NO_3^- was also similar between blocks, as was Cu. Unlike at Becker, there was no one block with a majority of differences relative to the others.

Discussion

This study investigated the effects of N fertilization and cultivar on switchgrass rhizosphere bacterial community structure at two locations in Minnesota. Switchgrass characteristics (yield, tissue N concentration, and root biomass) were also examined to determine whether relationships between plant growth and the rhizobacterial community could be found. Switchgrass yield and N concentration were part of a larger study, and a full discussion of results can be found in Chapter 1 (this volume). Yield and tissue N results from this study, however, support many others that have demonstrated differences in switchgrass productivity and nutrient uptake as a function of site characteristics (e.g. Casler and Boe 2003; Guretzky et al. 2011; Mangan et al. 2011). Switchgrass yield was not correlated with increasing relative abundance of any bacterial orders, and any orders correlated with tissue N were also correlated with soil NO_3^--N.

Similar to aboveground biomass, root biomass differed by location, and was ~30% less at Becker than at Lamberton. This finding is similar to results from Kulmatiski et al. (2017), who found soil texture to be a greater determinant of root biomass than precipitation, with greater root biomass in fine-textured soils relative to coarse-textured soils. Root biomass increased in response to N fertilization at Becker but not at Lamberton. Several studies have demonstrated mixed results in switchgrass root biomass response to N fertilization: Ma et al. (2000) and Jung and Lal (2011) found no response to N fertilization, while Heggenstaller et al. (2009) found 140 kg N ha^{-1} maximized root biomass growth relative to 0, 65, or 220 kg N ha^{-1}. Root biomass, however, was not correlated with rhizosphere community structure at either location.

Similar to many other studies, we anticipated that the rhizosphere effect on selecting and shaping bacterial communities would differ between our two locations (Ulrich and Becker 2006; da Jesus et al. 2010; Schreiter et al. 2014; Fernandez et al. 2016). Differences in cropping history and edaphic qualities such as soil nutrient status, water availability, and pH can influence root exudate quantity and composition which, in turn, affects the rhizosphere community structure (Brimecombe et al. 2001). Furthermore, plant interactions with the soil microbiome can influence root exudate composition even in the absence of known plant stress factors (Neumann et al. 2014). Because of anticipated differences between sites, we elected not to make statistical comparisons between locations, instead focusing on within-site differences in treatments while acknowledging similarities in response at each location.

There were minimal differences in community diversity (Shannon index) and no differences in community richness as a function of treatment at either location. These results are not unexpected, given that microbial diversity has been shown to be similar under differing management regimes, even as community structure differs (Wu et al. 2008; Fernandez et al. 2016). Of the twelve most abundant orders at each location, only six were shared between sites, similar to results of many other studies, indicating that rhizosphere community bacterial populations are shaped primarily by existing soil characteristics and microbial communities (Bulgarelli et al. 2012; Arenz et al. 2014; Hargreaves et al. 2015; Fernandez et al. 2016). Three of the orders shared between locations, *Burkholderiales*, *Rhizobiales*, and *Sphingomonadales* were also found to be enriched in switchgrass the rhizosphere relative to bulk soil in a study of root exudates under unfertilized conditions (Mao et al. 2014a).

Community structure was also shaped by cultivar, similar to results from studies examining other species (Germida and Siciliano 2001; Knox et al. 2014; Winston et al. 2014; Wen et al. 2017). Differences between cultivars were observed only in minor orders (<1.5% of sequence reads), however, and may not overwhelmingly influence plant-microbe dynamics or nutrient cycling relative to other cultivars. The effect of cultivar was stronger at Becker than at Lamberton, where treatment effects had less influence over the variation in community structure than did soil/plant parameters, likely as a result of high soil fertility and/or soil texture. Differences in bacterial orders as a function of cultivar may also have been influenced by N fertilization, but the taxonomic diversity was too great to resolve discrete patterns related to the cultivar × N rate interaction. For example,

at Becker, *Sphingobacteriales* accounted for 1.5% of sequence reads, and was one of three orders greater in relative abundance in the 'Liberty' rhizosphere relative to other cultivars. However, *Sphingobacteriales* was also positively correlated with tissue N concentration and soil NO_3^-, both of which were generally greater in fertilized 'Liberty' relative to other cultivars. 'Liberty' was the only cultivar to exhibit differences in bacterial orders at both locations, likely as a function of variations in exudate composition that can exist between cultivars of the same species (Christensen-Weniger et al. 1992). However, the differing taxa were not consistent between locations, which is not unexpected given the extremely high diversity observed at both locations; functional traits of bacterial communities have been shown to be conserved, despite differences in diversity resulting from functional redundancy among bacteria (Lozupone et al. 2012).

Community shifts with N fertilization, however, can have important implications for nutrient cycling and C sequestration. Nitrogen enrichment can reduce need for microbes to "mine" recalcitrant organic matter for N, thereby fostering increased labile C decomposition and decreased recalcitrant C composition, leading to increased C sequestration in soils (Craine et al. 2007; Ramirez et al. 2012). We found that N fertilization shaped rhizobacterial community structure at both locations, consistent with other studies (e.g. Ramirez et al. 2012; Fernandez et al. 2016; Zhu et al. 2016). We also found the most pronounced shifts in community structure in response to N in the low OM soil at Becker (62 orders) and less pronounced shifts in the Lamberton loam soil with greater OM (15 orders), consistent with results from Bakker et al. (2015) and Ramirez et al. (2012). At both locations there were 12 orders exhibiting an identical response as a

function of N fertilization, including orders containing genera that are specifically involved in N cycling, similar to Zhu et al. (2016). Specifically, we found increases in *Nitrosomonadales,* which contains the ammonia-oxidizing bacteria (AOB) genus *Nitrosomonas*, and increases in *Rhodocyclales*, which contains several genera of plant-associated diazotrophs as well as several genera of denitrifying bacteria (Oren 2014). The increased abundance of anaerobic denitrifying bacteria, even in excessively-drained soil, is not unexpected: roots generally promote denitrification in the rhizosphere through oxygen consumption and creation of localized anaerobic conditions, while simultaneously providing a C substrate for denitrifying bacteria (Philippot et al. 2013). We also found increased abundance in *Rhizobiales* as a function of N addition at Lamberton, which is somewhat contrary to results from Fernandez et al. (2016), who found a decrease in rhizosphere *Rhizobiales* with soluble N addition, but only at one of three locations. However, these differences could be a function of species sorting dynamics in the rhizosphere or a function of variability in soil edaphic factors at each location. For example, N addition alone has been shown to decrease rhizobia abundance in soil with low OM (Coelho et al. 2009), but N-P-K fertilizers can enhance rhizobial abundance by eliminating nutrient deficiencies (Germida 1988; Simonsen et al. 2015). It is possible that OM content, other soil characteristics, or legacy effects from previous crops (including soybeans) at Lamberton facilitated the increase in *Rhizobiales* abundance with fertilizer N.

Several soil physiochemical parameters were correlated with greater relative abundance in orders at both locations. While there were few similarities between sites, *Burkholderiales* abundance was negatively correlated with pH at both locations.

Burkholderiales contain the plant-associated genera *Burkholderia*, which has a known tolerance for acidic soil conditions (Stopnisek et al. 2014). It is not unexpected that most other associations between orders and soil physiochemical parameters were different at each location, given that soil/plant parameters explained the majority of the variation in community structure. Consistent with findings from many other studies, these results indicate that the differences in soil characteristics have greater influence on shaping rhizobacterial communities than do treatment effects (e.g. Bulgarelli et al. 2012; Bakker et al. 2013; Hargreaves et al. 2015).

While many other studies have examined variability in soil bacterial communities as a function of soil characteristics across locations (Girvan et al. 2003; Fierer and Jackson 2006; Ulrich and Becker 2006; Ramirez et al. 2012; Liang et al. 2012), few address potential effects of spatial variability at a single site within an experimental design such as a randomized complete block. Spatial variability in microbiological communities is directly affected by spatial variability in physiochemical parameters in soil, even across a single field with common soil type (Ettema and Wardle 2002; Baker et al. 2009; Bakker et al. 2013). While considering within-site spatial variability was not a stated objective of this study, we observed differences in microbial community structure as a function of location within plot, as designated by experimental block. We also found differences in nearly every observed soil physiochemical parameter among blocks at each location. At Lamberton, there were also differences in bacterial richness and diversity as a function of block. However, differences in taxa occurred in very minor classified orders (< 0.1% of OTUs) at each location.

While our results cannot speak to the relative influence of soil edaphic characteristics and previous cropping history on spatial variability in soil physiochemical parameters or bacterial community composition, it is interesting to note that cropping histories at each location were quite different. At Becker, only one crop (cereal rye) had been planted annually since at least 1995 and all amendments were similarly applied to the entire plot. At Lamberton, previous cropping history included a corn/soybean rotation with amendments applied to the entire plot (2009-2012) and a corn/soybean tillage trial previous to that, with amendments applied differentially to blocks (2004-2008). Further research is necessary to clarify the impact and duration of legacy effects from prior management on contemporary rhizosphere bacterial communities. Legacy effects may have great importance for assessing changes in nutrient cycling, plant-microbe interactions, or even for plant breeding with intent to alter rhizosphere microbial communities.

Conclusions

We examined the effects of N fertilization and cultivar on switchgrass rhizosphere bacterial community structure at two locations in Minnesota, each with contrasting soil characteristics. We found that species sorting dynamics and the resulting communities were generally different at each location as a result of soil characteristics. Soil and plant characteristics explained a greater proportion of the variability in community structure than did treatment effects at both locations, although treatments did contribute to species sorting at both locations. Cultivar affected community structure at both locations, with

most differences in bacterial orders found in rhizosphere soil from ‘Liberty’. Cultivar effects were confined to minor taxa that were not consistent between locations, indicating that the high bacterial diversity in soils confounds the resolution of universal trends shaping community composition. We also found that N fertilization affected bacterial orders that include taxa integral to N cycling, and many of these orders were consistent between locations. Finally, we explored the effect of spatial variability in soil edaphic characteristics on switchgrass rhizosphere communities as a function of experimental block. While community differences were evident between blocks, they were few and did not impact any of the most abundant taxa. Future research is warranted, however, to better understand the effects of spatial variability on metagenomic studies in agricultural experiments. Overall, the lack of a core switchgrass rhizosphere microbiome, as shaped by treatment effects, is not unexpected, given the great diversity and functional redundancy in bacterial communities.

Table 2-1. Locations, soil series descriptions, and pre-establishment soil test results for experimental plots.

Site	Location	Soil type	Bray P	Extractable K	Organic matter	Water pH
			ppm		%	
Becker	45.39N, 93.88W	Hubbard-Mosford complex loamy sand (Sandy, mixed, frigid Entic (Hubbard) and Typic (Mosford) Hapludolls)	25.3	53.0	1.3	6.4
Lamberton	44.24N, 95.30W	Amiret-Swanlake loams (fine-loamy, mixed, superactive, mesic Calcic Hapludolls (Amiret) and fine-loamy, mixed, superactive, mesic Typic Calciudolls (Swanlake))	13.0	140.0	4.9	5.3

Table 2-2. Cultivar × nitrogen rate interaction effects on nitrogen concentration in switchgrass at Becker, MN. Values sharing the same letter did not differ significantly at $\alpha = 0.05$.

Cultivar	N rate	N concentration
	kg ha^{-1}	%
Liberty	0	1.04 b
	112	1.79 a
Shawnee	0	0.96 b
	112	1.68 a
Sunburst	0	1.04 b
	112	1.22 b

Table 2-3. Cultivar and nitrogen rate effects on nitrate and ammonia in soil taken from core samples containing switchgrass roots. At Becker, MN, the cultivar × nitrogen rate interaction was significant; at Lamberton, MN, main effects of cultivar and nitrogen rate were significant.

	Cultivar	N	NO_3^-	NH_4^+
Becker		kg ha^{-1}	ppm	
	Liberty	0	5.03 b	7.34
		112	22.66 a	6.65
	Shawnee	0	4.98 b	7.26
		112	9.37 b	6.53
	Sunburst	0	3.53 b	7.65
		112	8.37 b	7.72
Lamberton	Liberty		11.57 a	26.23
	Shawnee		7.16 b	24.49
	Sunburst		6.35 b	28.27
		0	4.69 b	26.14
		112	12.04 a	26.53

Table 2-4. Nitrogen and cultivar effects on community diversity (Shannon index) in rhizosphere soil samples at Becker and Lamberton, MN. Values sharing the same letter did not differ significantly at $\alpha = 0.05$.

Cultivar	N	Becker	Lamberton
	kg ha^{-1}	Shannon	
Liberty	0	10.88 a	10.24
	112	10.46 b	10.11
Shawnee	0	10.97 a	10.20
	112	10.85 a	10.14
Sunburst	0	10.77 a	10.06
	112	10.79 a	10.13

Table 2-5. Bacterial orders that are more abundant at both Becker and Lamberton as a function of nitrogen application based on linear discriminant analysis (LDA).

N	Class Order	Becker	Lamberton
kg ha^{-1}		LDA score (log 10)	
100	*Sphingobacteriia Sphingobacteriales*	3.531	2.942
	Betaproteobacteria Rhodocyclales	3.268	2.248
	Flavobacteriia Flavobacteriales	2.819	2.525
	Betaproteobacteria Nitrosomonadales	2.546	2.472
	TM7-3 Unclassified	2.412	2.660
0	*Pla3 Unclassified*	-2.191	-2.002
	Deltaproteobacteria NB1-j	-2.216	-2.447
	PBS-25 Unclassified	-2.274	-2.054
	Mollicutes Anaeroplasmatales	-2.520	-2.892
	PRR-12 Sediment 1	-2.637	-2.427
	Gemmatimonadetes KD8-87	-2.690	-2.243
	Pedosphaerae Pedosphaerales	-2.916	-3.554

Table 2-6. Spearman correlation values for soil and plant parameters associated with bacterial orders at Becker, MN. *P* values are shown in parentheses and significant values ($\alpha < 0.05$, adjusted to $\alpha < 0.002$ using Bonferroni correction for multiple comparisons) are bolded.

Plant/soil parameter	*Actinomycetales*	*Burkholderiales*	*Chthoniobacterales*	*Acidobacteria-6 iii1-15*	*Rhizobiales*	*Chloracidobacteria RB41*	*Pirellulales*	*Pseudomonadales*	*Sphingobacteriales*
Yield	-0.193 (0.104)	0.000 (0.998)	-0.168 (0.158)	-0.201 (0.090)	-0.107 (0.372)	-0.173 (0.145)	-0.238 (0.044)	0.224 (0.059)	-0.016 (0.892)
Tissue N	0.143 (0.229)	**0.421 (<0.001)**	-0.037 (0.759)	-0.233 (0.049)	0.068 (0.571)	-0.230 (0.052)	-0.198 (0.096)	**0.415 (<0.001)**	**0.478 (<0.001)**
Root biomass	-0.107 (0.372)	-0.067 (0.573)	0.018 (0.877)	-0.170 (0.153)	-0.128 (0.284)	-0.089 (0.456)	-0.146 (0.220)	0.061 (0.608)	0.072 (0.547)
Moisture	-0.060 (0.614)	0.012 (0.919)	0.177 (0.137)	0.191 (0.108)	-0.034 (0.774)	0.300 (0.011)	0.271 (0.022)	-0.163 (0.170)	-0.026 (0.828)
pH	-0.269 (0.022)	**-0.426 (<0.001)**	-0.247 (0.037)	0.247 (0.037)	-0.224 (0.059)	0.172 (0.149)	0.140 (0.239)	-0.051 (0.673)	**-0.377 (0.001)**
NH4	-0.274 (0.02)	-0.142 (0.233)	-0.115 (0.337)	0.027 (0.821)	-0.144 (0.226)	0.036 (0.763)	0.235 (0.047)	-0.163 (0.170)	-0.214 (0.072)
NO3	0.238 (0.045)	**0.421 (<0.001)**	-0.013 (0.916)	-0.158 (0.183)	0.222 (0.061)	-0.146 (0.220)	-0.174 (0.144)	**0.360 (0.002)**	**0.395 (0.001)**
P	-0.209 (0.078)	0.091 (0.446)	0.192 (0.106)	0.307 (0.009)	-0.127 (0.287)	0.318 (0.007)	**0.485 (<0.001)**	-0.069 (0.565)	0.012 (0.920)
Fe	0.197 (0.097)	0.248 (0.036)	**0.378 (0.001)**	0.152 (0.202)	0.229 (0.053)	0.267 (0.024)	0.195 (0.100)	-0.113 (0.343)	0.201 (0.091)
Mn	0.211 (0.076)	0.246 (0.037)	0.321 (0.006)	0.240 (0.043)	0.199 (0.094)	0.333 (0.004)	0.225 (0.058)	0.092 (0.443)	0.220 (0.063)
Zn	0.201 (0.090)	0.185 (0.119)	0.226 (0.056)	0.195 (0.101)	0.201 (0.090)	0.283 (0.016)	0.206 (0.083)	-0.108 (0.364)	0.174 (0.143)
Cu	0.076 (0.527)	0.069 (0.565)	0.111 (0.351)	0.224 (0.058)	0.109 (0.362)	0.276 (0.019)	0.175 (0.141)	-0.058 (0.625)	0.033 (0.781)
Pb	0.312 (0.008)	0.228 (0.054)	0.347 (0.003)	0.261 (0.027)	**0.356 (0.002)**	**0.348 (0.003)**	0.159 (0.181)	-0.117 (0.326)	0.211 (0.076)
Ni	0.132 (0.268)	0.294 (0.012)	0.166 (0.163)	0.215 (0.070)	0.104 (0.383)	0.280 (0.017)	0.257 (0.029)	0.003 (0.981)	0.248 (0.036)
Cd	0.131 (0.270)	0.287 (0.015)	0.267 (0.024)	0.314 (0.007)	0.169 (0.156)	**0.391 (0.001)**	0.331 (0.005)	-0.002 (0.985)	0.281 (0.017)
Cr	-0.158 (0.186)	0.238 (0.044)	0.201 (0.091)	0.130 (0.277)	-0.170 (0.152)	0.125 (0.296)	**0.375 (0.001)**	0.042 (0.724)	0.204 (0.085)

Table 2-7. Spearman correlation values for soil and plant parameters associated with bacterial orders at Lamberton, MN. *P* values are shown in parentheses and significant values (based on $\alpha < 0.05$, adjusted to $\alpha < 0.002$ using Bonferroni correction for multiple comparisons) are bolded.

Plant/soil parameter	*Burkholderiales*	*Actinomycetales*	*Acidobacteriales*	*Sphingomonadales*	*Xanthomonadales*	*Chthoniobacterales*	*Rhizobiales*	*Saprospirales*
Yield	-0.040 (0.737)	0.024 (0.843)	0.004 (0.972)	0.171 (0.150)	0.096 (0.422)	-0.130 (0.276)	0.056 (0.642)	-0.030 (0.804)
Tissue N	0.157 (0.187)	-0.115 (0.337)	-0.119 (0.320)	-0.087 (0.468)	-0.097 (0.415)	**-0.375 (0.001)**	-0.116 (0.332)	-0.288 (0.015)
Root biomass	0.095 (0.428)	-0.07 (0.558)	-0.006 (0.963)	-0.085 (0.478)	0.005 (0.969)	-0.113 (0.344)	-0.011 (0.924)	-0.026 (0.825)
Moisture	-0.135 (0.259)	0.128 (0.283)	0.058 (0.628)	0.196 (0.099)	0.164 (0.169)	0.239 (0.044)	0.131 (0.270)	0.274 (0.020)
pH	**-0.515 (<0.001)**	-0.035 (0.768)	-0.176 (0.139)	0.092 (0.441)	-0.067 (0.574)	**0.562 (<0.001)**	0.022 (0.853)	0.274 (0.020)
NH4	-0.233 (0.050)	0.082 (0.490)	-0.099 (0.406)	**0.405 (<0.001)**	0.118 (0.324)	**0.388 (0.001)**	0.129 (0.279)	0.281 (0.017)
NO3	0.244 (0.039)	0.204 (0.086)	0.069 (0.563)	0.197 (0.098)	0.203 (0.087)	-0.220 (0.064)	0.197 (0.097)	0.018 (0.881)
P	0.096 (0.420)	0.132 (0.270)	0.097 (0.415)	0.303 (0.010)	0.196 (0.099)	0.019 (0.875)	0.08 (0.503)	0.246 (0.038)
Fe	-0.183 (0.124)	0.126 (0.290)	0.023 (0.849)	0.311 (0.008)	0.209 (0.078)	**0.354 (0.002)**	0.154 (0.195)	0.280 (0.017)
Mn	**0.428 (<0.001)**	0.198 (0.096)	0.253 (0.033)	0.230 (0.052)	0.265 (0.025)	-0.330 (0.005)	0.185 (0.119)	0.040 (0.741)
Zn	0.003 (0.978)	0.245 (0.038)	0.144 (0.226)	**0.396 (0.001)**	0.310 (0.008)	0.237 (0.045)	0.244 (0.039)	**0.408 (<0.001)**
Cu	-0.070 (0.561)	0.351 (0.003)	0.227 (0.056)	0.302 (0.010)	0.314 (0.008)	**0.373 (0.001)**	0.346 (0.003)	**0.387 (0.001)**
Pb	-0.151 (0.205)	0.217 (0.067)	0.032 (0.790)	**0.379 (0.001)**	0.247 (0.037)	**0.409 (<0.001)**	0.210 (0.077)	**0.361 (0.002)**
Ni	**0.360 (0.002)**	**0.453 (<0.001)**	**0.368 (0.002)**	**0.363 (0.002)**	**0.470 (<0.001)**	-0.055 (0.643)	**0.439 (<0.001)**	0.301 (0.011)
Cd	0.183 (0.124)	0.330 (0.005)	0.276 (0.019)	**0.423 (<0.001)**	**0.408 (<0.001)**	0.080 (0.502)	0.321 (0.006)	0.344 (0.003)
Cr	**0.493 (<0.001)**	0.088 (0.461)	0.232 (0.050)	0.134 (0.261)	0.213 (0.072)	**-0.520 (<0.001)**	0.092 (0.443)	-0.172 (0.149)

Table 2-8. Block effects on diversity and richness indices in rhizosphere soil samples evaluated by block at Becker and Lamberton, MN: A) Shannon diversity index, B) Chao 1 richness estimate. Values sharing the same letter down a column did not differ significantly at $\alpha = 0.05$.

A	Block	Becker	Lamberton
		Shannon	
	1	10.77	9.73 c
	2	10.78	10.41 a
	3	10.83	10.38 a
	4	10.76	10.07 b
B		Chao1	
	1	18352 a	12164 b
	2	16904 b	13160 a
	3	18111 a	13274 a
	4	17790 ab	12340 b

Table 2-9. Physiochemical parameters in bulk soil taken from core samples containing switchgrass roots at A) Becker and B) Lamberton, MN, analyzed by field location (block).

Location	Block	Moisture	pH	NH_4^+	NO_3^-	P	Fe	Mn	Zn	Cu	Pb	Ni	Cd	Cr
		%		ppm										
Becker	1	6.74 b	5.96 a	7.41 b	9.81	30.22 a	16.87 bc	11.14 b	0.54 c	0.17 c	0.35 b	0.33 b	$5.04E^{-2}$ b	$3.50E^{-2}$ a
	2	6.32 c	5.76 b	6.58 c	9.70	17.03 c	17.82 b	12.67 b	0.70 b	0.21 b	0.41 b	0.30 b	$5.09E^{-2}$ b	$1.00E^{-2}$ b
	3	6.48 bc	5.94 a	6.22 c	8.29	17.67 c	16.18 c	12.80 b	0.53 c	0.22 b	0.37 b	0.32 b	$4.80E^{-2}$ b	$1.00E^{-2}$ b
	4	8.03 a	5.87 ab	8.56 a	8.13	27.28 b	22.32 a	18.81 a	1.00 a	0.28 a	0.60 a	0.58 a	$9.46E^{-2}$ a	$1.36E^{-2}$ b
	F statistic	37.4	2.5	12.5	0.1	54.2	26.3	29.8	45.5	65.0	15.2	20.7	103.3	26.5
	P value	<0.001	0.044	<0.001	0.947	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Lamberton	1	15.52 b	4.98 b	22.95 c	10.12	12.06 b	152.03 b	96.73 a	1.09 bc	1.21	1.59 b	4.15 a	$1.58E^{-2}$ a	$2.45E^{-2}$ a
	2	17.82 a	5.17 a	29.14 b	7.73	19.11 a	176.69 a	92.54 b	1.20 a	1.26	1.75 a	4.01 b	$1.59E^{-2}$ a	$2.12E^{-2}$ ab
	3	16.00 b	5.14 a	33.52 a	8.60	12.33 b	169.71 a	80.90 b	1.14 ab	1.24	1.78 a	3.81 c	$1.52E^{-2}$ a	$2.03E^{-2}$ ab
	4	14.62 b	5.02 b	19.73 c	6.99	12.11 b	155.71 b	80.84 b	1.05 c	1.18	1.61 b	3.67 d	$1.33E^{-2}$ b	$1.73E^{-2}$ b
	F statistic	4.4	10.2	21.4	0.9	4.5	7.0	9.0	6.5	2.2	9.6	18.9	11.0	3.4
	P value	0.007	<0.001	<0.001	0.449	0.006	<0.001	<0.001	<0.001	0.102	<0.001	<0.001	<0.001	0.024

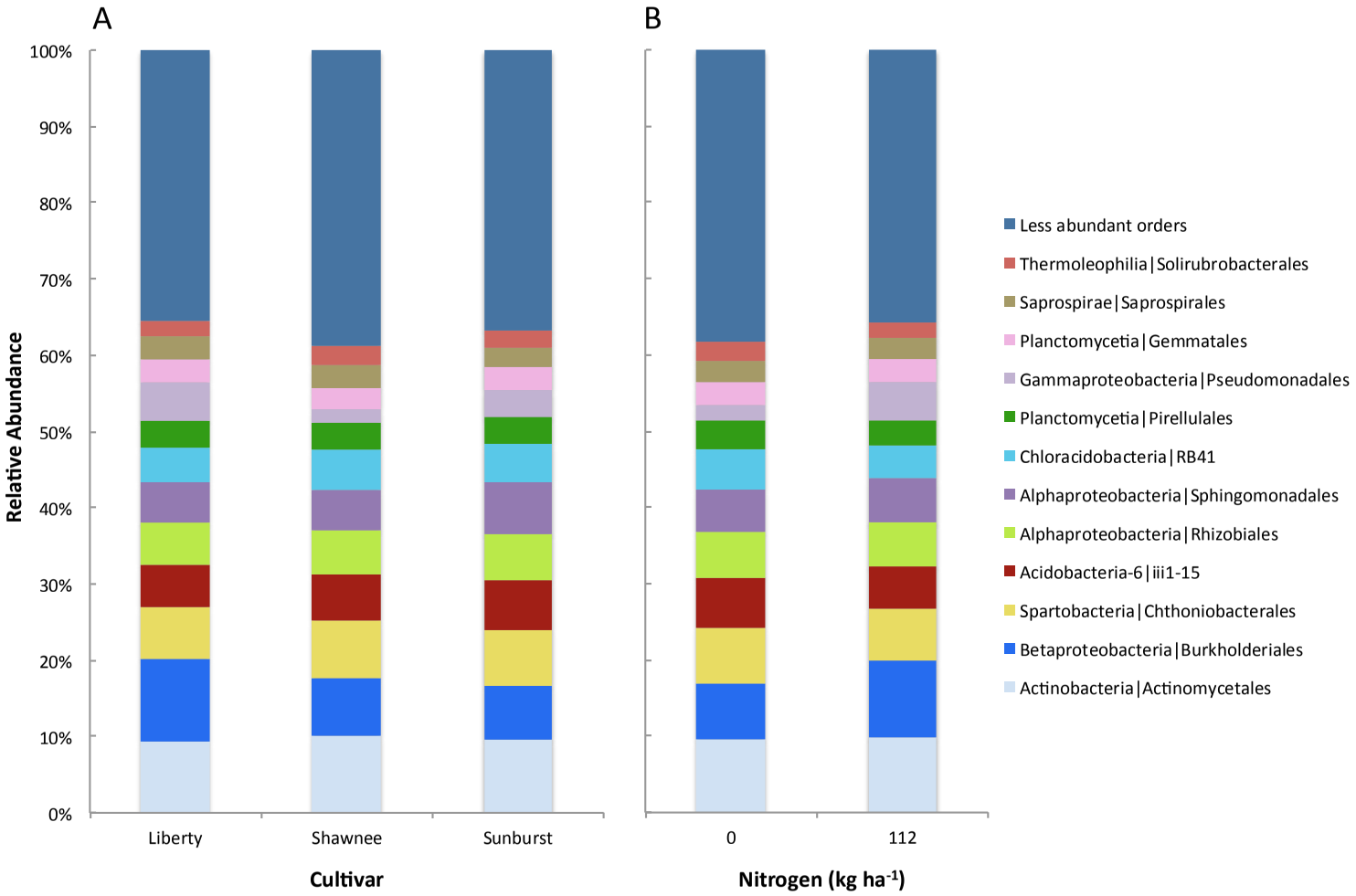


Figure 2-1. Distribution of taxonomic orders by A) cultivar and B) nitrogen application rate at Becker, MN.

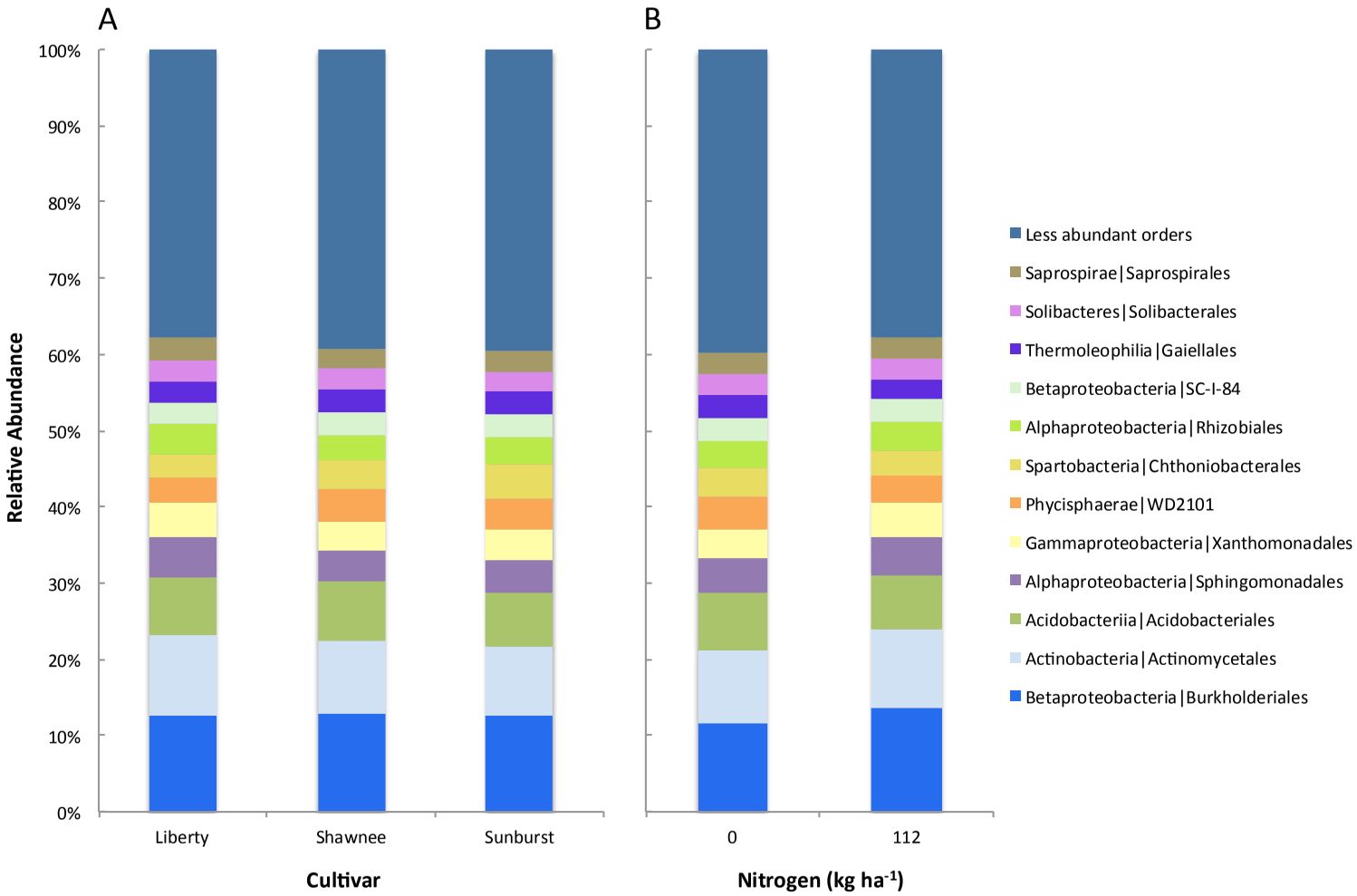


Figure 2-2. Distribution of taxonomic orders by A) cultivar and B) nitrogen application rate at Lamberton, MN.

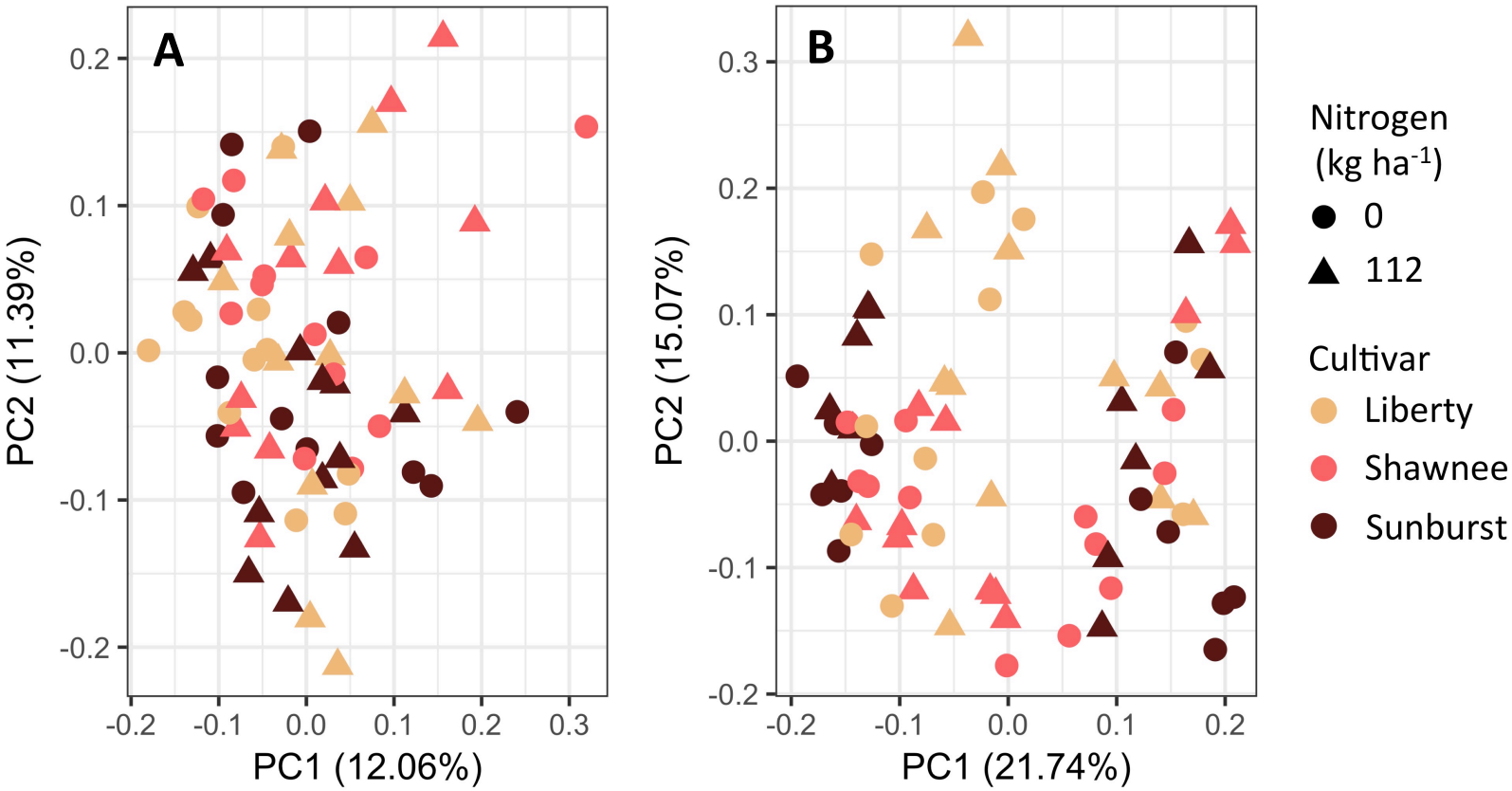


Figure 2-3. Principal coordinate analysis (based on Bray-Curtis distance matrix) of switchgrass rhizosphere bacteria samples, identified by nitrogen fertilization and cultivar. A) Becker and B) Lamberton, MN.

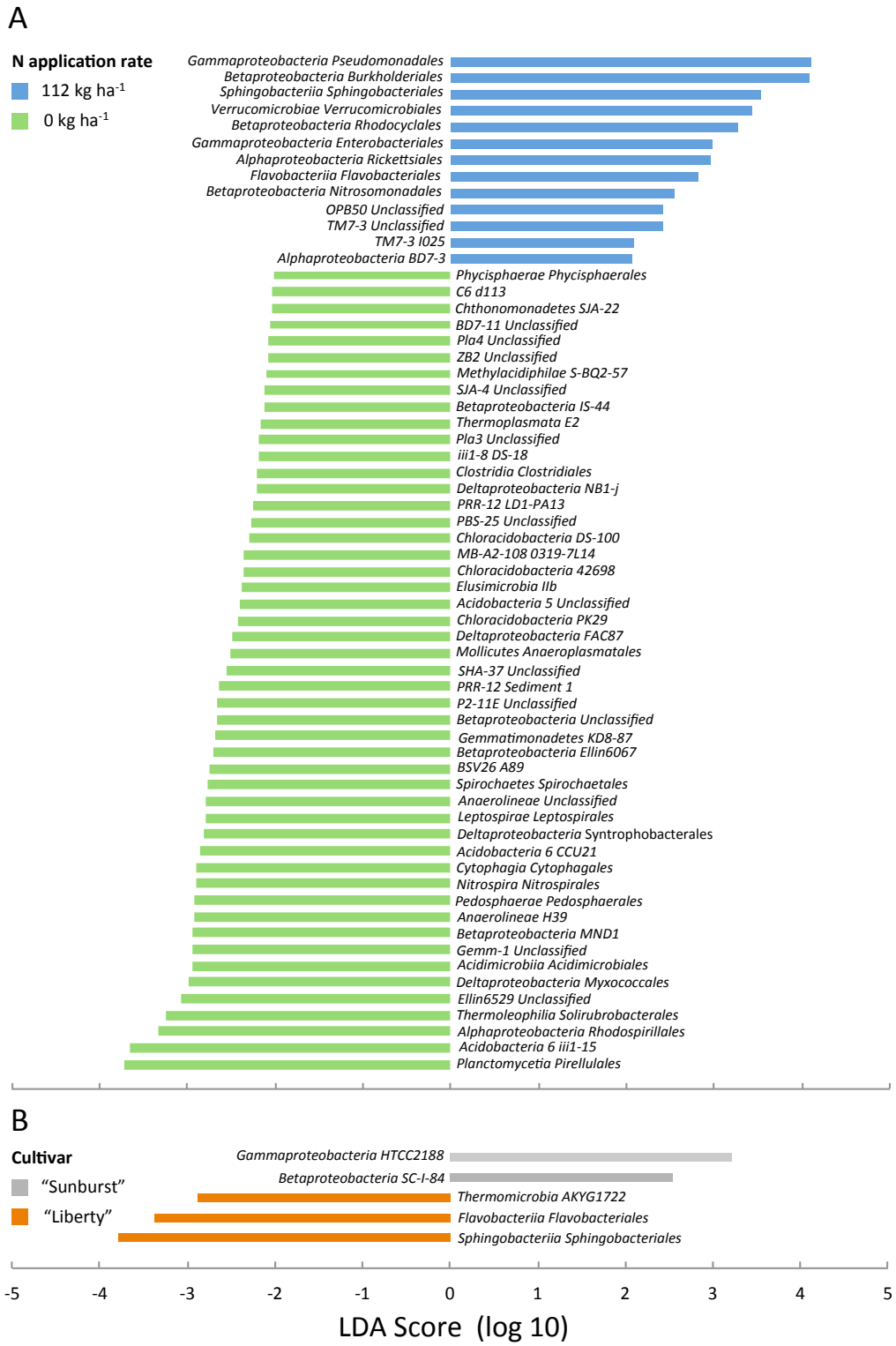


Figure 2-4. Linear discriminant analysis by bacterial order at Becker, MN. Differences shown are within A) nitrogen application rate and B) cultivar treatments.

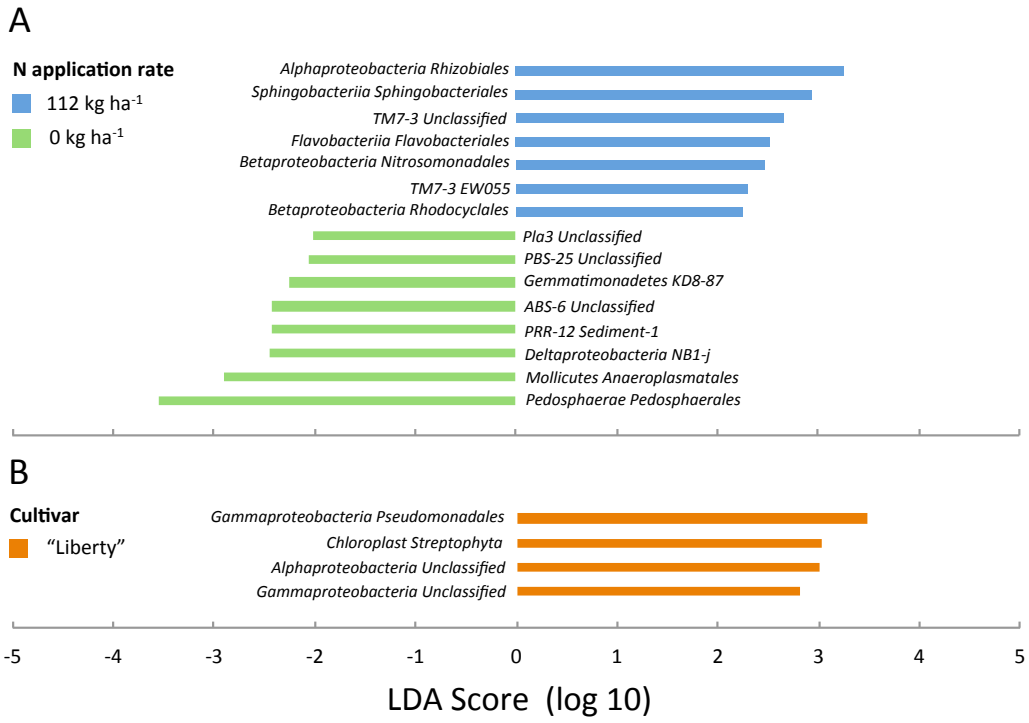


Figure 2-5. Linear discriminant analysis by bacterial order at Lamberton, Minnesota. Differences shown are within A) nitrogen application rate and B) cultivar.

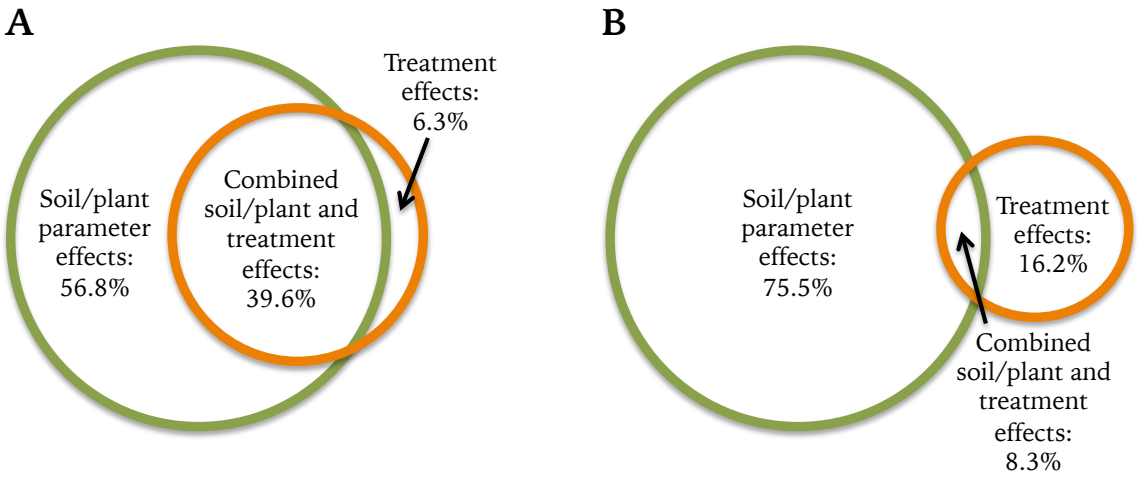


Figure 2-6. Variance partitioning of relative abundance of bacterial orders as a function of soil and plant physiochemical parameters, treatment effects, and combined soil/plant and treatment effects at Becker (A) and Lamberton (B).

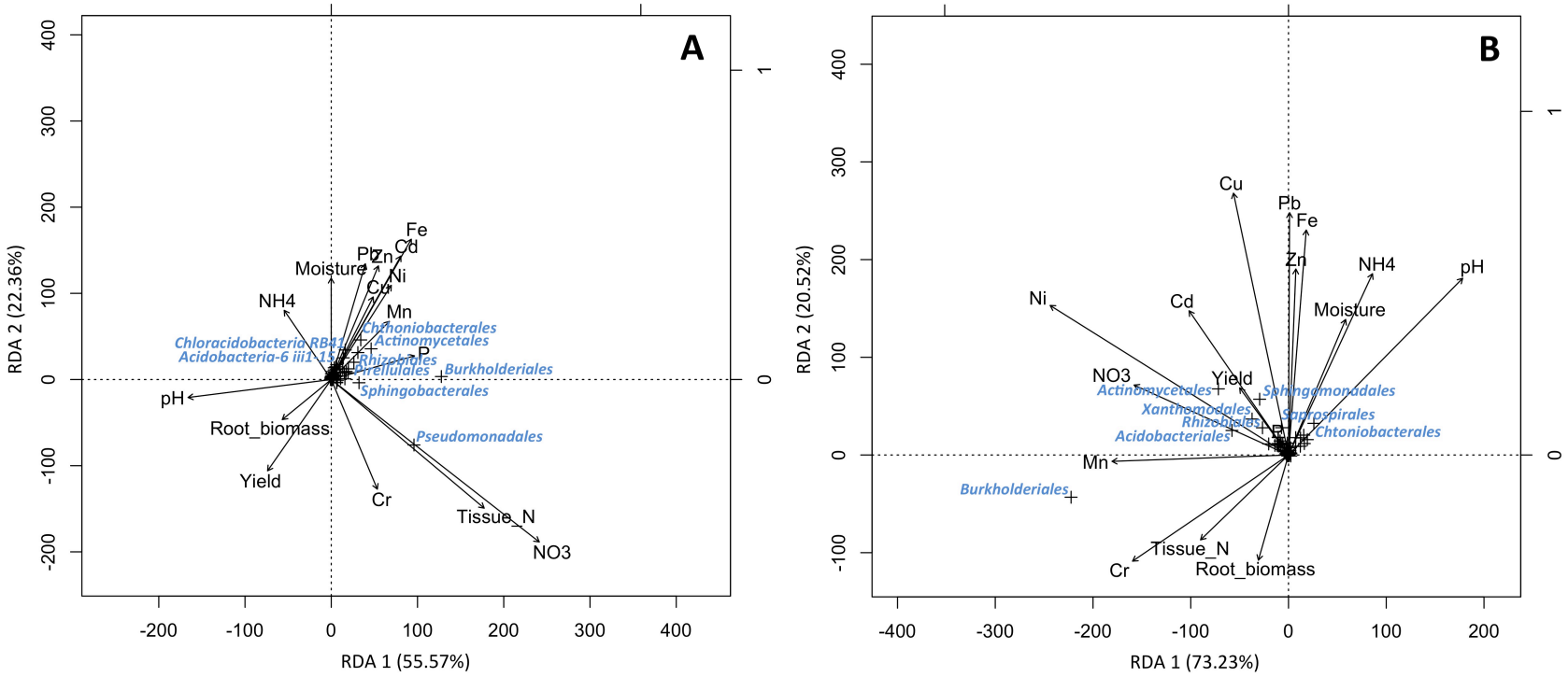


Figure 2-7. Redundancy analysis showing variance in relative abundances of bacterial orders as explained by plant and soil parameters at A) Becker, and B) Lamberton, MN.

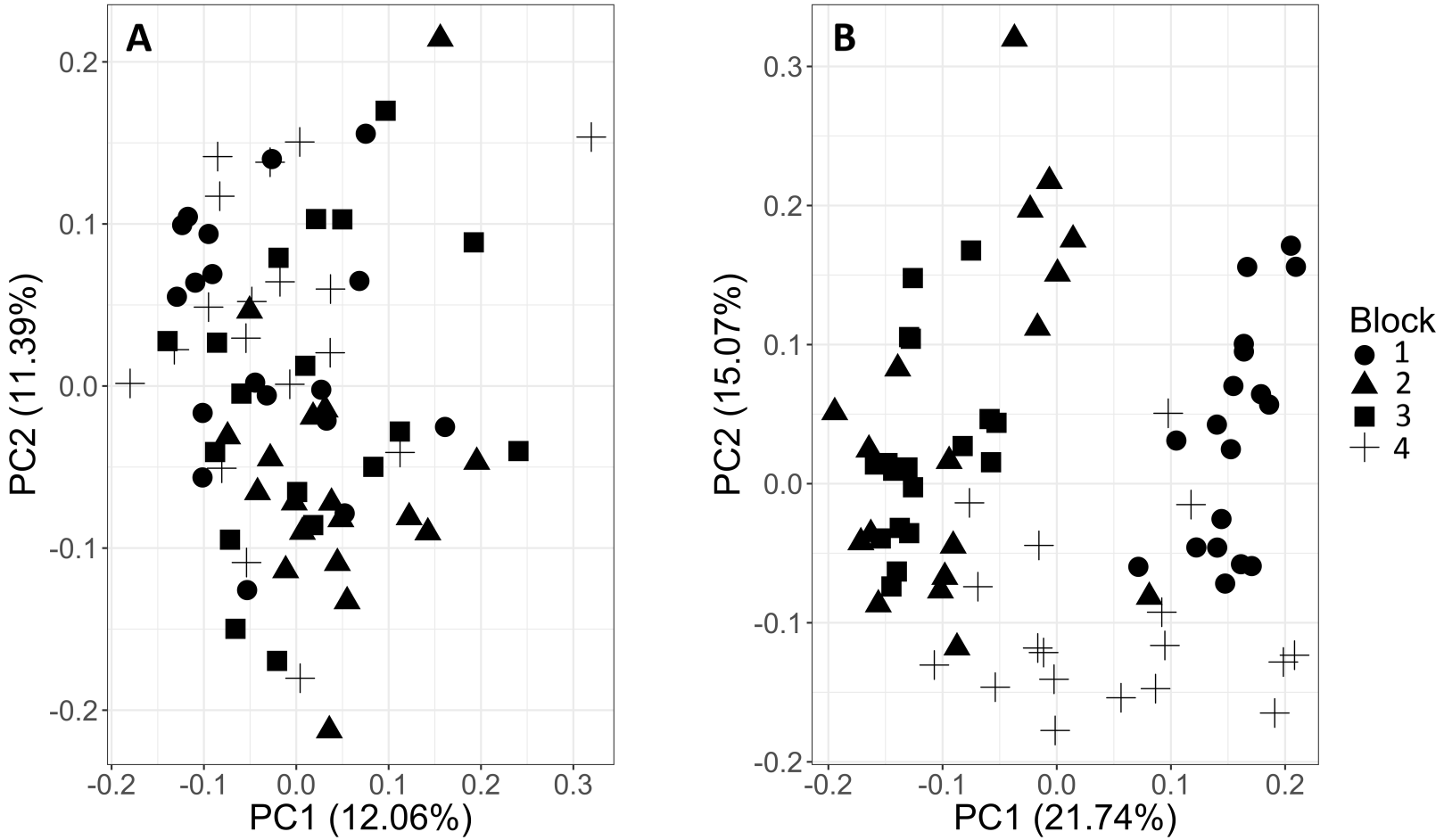


Figure 2-8. Principal coordinate analysis (based on Bray-Curtis distance matrix) of switchgrass rhizosphere bacteria samples, identified by experimental block within each plot. A) Becker and B) Lamberton, MN.

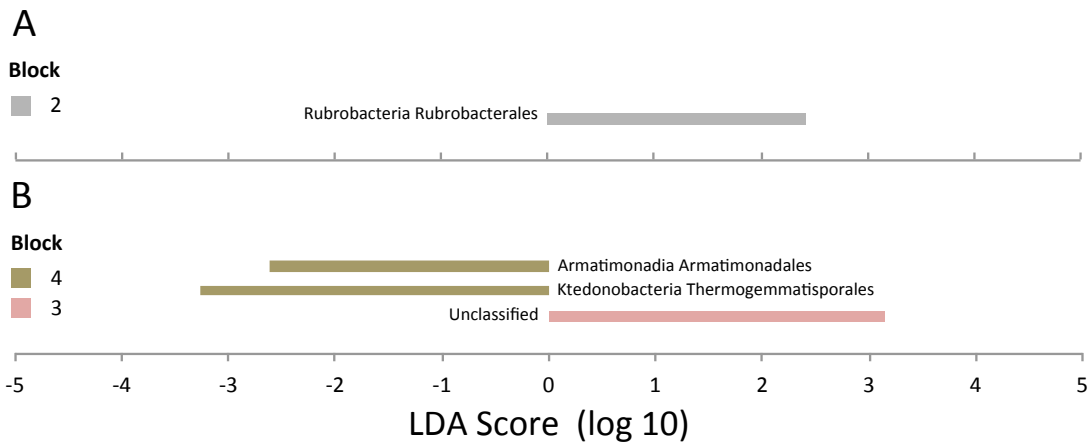


Figure 2-9. Linear discriminant analysis by bacterial order. Differences shown are within block at A) Becker and B) Lamberton, MN.

Chapter 3 – Cultivar and phosphorus fertilization effects on switchgrass biomass yield, phosphorus removal, and rhizosphere microflora

Abstract

Switchgrass (*Panicum virgatum* L.) is a native perennial grass identified as a promising biofuel crop for production on marginal agricultural lands. As such, research into switchgrass fertility and the switchgrass rhizosphere microbiome has been ongoing in an effort to increase sustainability in production. In this study, we examined the effects of cultivar and phosphorus (P) fertilization on biomass yield, phosphorus removal, and rhizosphere bacterial and fungal community structure in three switchgrass cultivars: 'Sunburst', 'Shawnee', and 'Liberty', the first lowland bioenergy switchgrass adapted to USDA hardiness zones 4, 5, and 6. Biomass increased linearly in response to increasing P application on a low to medium soil test P clay loam soil. Applying 19.6 and 39.1 kg P ha^{-1}, prior to establishment, provided average post-frost biomass yields of 10.1 and 10.3 Mg ha^{-1} yr^{-1}, respectively, over three years. 'Shawnee' was more productive than 'Liberty' or 'Sunburst' (11.3, 10.2, and 8.6 Mg ha^{-1} yr^{-1}, respectively). While cultivar was shown to influence both bacterial and fungal community structure in the rhizosphere, there were few consistent differences in taxa among cultivars. Phosphorus fertilization did not affect community structure among bacteria or fungi, despite a known switchgrass association with arbuscular mycorrhizal fungi for nutrient – particularly P – acquisition. The inability to detect fungal community differences as a function of P may be a result of known shortcomings in fungal sequencing, analyses, and taxonomy identification.

Overall, our results indicate that while the rhizosphere effect does influence bacterial and fungal community structure, existing soil physiochemical parameters explain a greater proportion of variability in the rhizosphere community than do treatment effects.

Introduction

Switchgrass (*Panicum virgatum* L.) is considered a desirable bioenergy crop based on its broad geographic range and high yield potential, particularly on marginal lands (Vogel et al. 2002; Mitchell et al. 2008). While switchgrass yield generally responds positively to nitrogen (N) fertilization (e.g. Vogel et al. 2002; Mulkey et al. 2008), limited information exists regarding yield response to phosphorus (P) fertilizer on soils of varying soil P test levels (Kering et al. 2012). Moreover, the effect of P fertilization is often confounded by significant N × P interactions (Rehm 1984; Rehm 1990; Brejda 2000) and by the symbiotic relationships formed with arbuscular mycorrhizal (AM) fungi that enhance the ability of warm-season grasses to grow on low-P soils (Hetrick et al. 1990; Brejda et al. 1993). Understanding P needs in switchgrass is essential to promoting switchgrass as a biofuel crop, because inadequate P nutrition can have adverse affects on overall biomass yield (Sutton et al. 1983; Kering et al. 2012). In particular, ensuring adequate P at germination may help to more rapidly establish switchgrass by promoting root and shoot growth (Morris et al. 1982; Römer and Schilling 1986) and reducing the length of time that bare soil is exposed to erosion.

The total amount of P in soils, however, often has little or no relationship to plant-available P: a complicated suite of pH-dependent precipitation and dissolution reactions,

adsorption and desorption activity, and microbial immobilization and mineralization governs inorganic P availability for plant uptake (Havlin et al. 2005). Plants, however, have adapted mechanisms for maximizing P acquisition, such as modifying root architecture and altering the composition of root exudates to increase mobility of soluble P sources (reviewed by Richardson et al. 2011). These changes, in turn, can alter the rhizosphere community structure, which is determined largely by plant root morphology and root exudate composition (Philippot et al. 2013) through the selection of microflora available from the preexisting population in the soil (Berg and Smalla 2009; Bulgarelli et al. 2012). Phosphorus acquisition by plants can be enhanced via symbiotic relationships with phosphate-solubilizing bacteria (Rodríguez and Fraga 1999) and AM fungi, members of the Glomeromycota phylum (Smith et al. 2008).

In agricultural systems, however, rhizosphere communities are greatly influenced by practices such as tillage and fertilizer/pesticide applications (Berg and Smalla 2009; Philippot et al. 2013). Nitrogen fertilization, for example, can directly alter root exudate quantity and composition, lower soil pH, and increase N availability for microbes, all of which have been shown to affect community structure, abundance, and function (Enwall et al. 2007; Ramirez et al. 2012; Geisseler and Scow 2014; Zhu et al. 2016). Less is known about the rhizosphere bacterial community response to P fertilization. Lagos et al. (2016) found significant changes in the ryegrass (*Lolium perenne*) rhizobacterial community composition in response to P fertilizer, while Jorquera et al. (2014) found that P fertilizer, applied alone or in conjunction with N, did not alter rhizobacterial composition in ryegrass. No studies to date have examined rhizobacterial response to P in switchgrass.

Several studies have examined the complex interactions between P fertilizer and AM fungi in the switchgrass rhizosphere (Hetrick et al. 1990; Brejda et al. 1993; Johnson 1998), but no studies to date have examined the broader fungal community response to P fertilization in the switchgrass rhizosphere.

A better understanding of differences in rhizosphere community structure among switchgrass cultivars may also be particularly important for sustainable production on marginal lands, where fertilization options may be limited and soil characteristics are highly variable. Breeding for characteristics such as yield or disease resistance can alter the natural selection of beneficial plant microbes in the rhizosphere, and domesticated plants may be less likely to benefit from rhizosphere microflora than their wild counterparts (Philippot et al. 2013; Gopal and Gupta 2016). Rhizosphere community structure and function have been found to vary with plant selection, both over time (Siciliano and Germida 1998; Germida and Siciliano 2001; Wen et al. 2017) and between contemporary cultivars (Inceoğlu et al. 2012; Knox et al. 2014; Winston et al. 2014). Switchgrass ecotype has been found to influence rhizosphere bacterial and fungal community structure under greenhouse conditions (Rodrigues et al. 2016), and bacterial communities were found to vary by cultivar under field conditions, but no bacterial orders were found to be consistently different between two soil types (Chapter 2, this volume). No studies to date have examined rhizosphere fungal community composition as shaped by switchgrass cultivar.

The responsible use of P fertilizer, in conjunction with a better understanding of plant-microbe interactions with P in the rhizosphere, is crucial for the development of

sustainable soil fertility and crop production (Johansson et al. 2004; Cordell and White 2014; Liang et al. 2016). Therefore, the objectives of this study were to: 1) Evaluate switchgrass biomass yield response to P fertilizer on a low to medium soil test P soil using two hardy upland forage cultivars, 'Shawnee' and 'Sunburst' (Vogel et al. 1996; Boe and Ross 1998), and 'Liberty', the first biomass cultivar (Vogel et al. 2014); 2) Examine the rhizobacterial community response to cultivar, P fertilization, and soil/plant physiochemical parameters using Illumina amplicon sequencing of the 16S rRNA gene; and 3) Examine the rhizosphere fungal community response to cultivar, P fertilization, and soil/plant physiochemical parameters using Illumina amplicon sequencing of the ITS1 (internal transcribed spacer) region over the course of two years.

Materials and methods

Site description and experimental design

We conducted this experiment at the University of Minnesota's Southwest Research and Outreach Center in Lamberton, MN (44.24N, 95.30W). Climate data for Lamberton (Table 3-1) were obtained from NOAA's Cooperative Network, via the Midwestern Regional Climate Center, cli-MATE (MRCC Application Tools Environment, http://mrcc.isws.illinois.edu/CLIMATE/, accessed 4/2017).

The soil at the experimental site was a Webster clay loam (Fine-loamy, mixed, superactive, mesic Typic Endoaquoll) with a 0-2% slope. The site was selected in 2012 based on pre-establishment soil tests (Table 3-2) indicating an average low soil test P value, according to University of Minnesota guidelines (Rehm et al. 1997). Pre-establishment soil samples were submitted to the University of Minnesota Research

Analytical Laboratory for testing. Briefly, soil pH was measured in a 1:1 (v/v) soil:water solution using a Mettler Toledo Seven-Multi pH meter with an InLab Routine Pro combination electrode (Mettler-Toledo International Inc., Columbus, OH), following Watson and Brown (1998); extractable soil phosphorus was measured in air-dried soil using the Bray P1 test (Frank et al. 1998) with molybdate-blue color development observed using a Brinkmann PC 900 probe colorimeter (Metrohm AG, Herisau, Switzerland); available soil potassium was extracted from air-dried soil with 1 M ammonium acetate and measured using a Perkin Elmer Analyst 100 atomic emission spectrometer (PerkinElmer Inc., Waltham, MA) (Warncke and Brown 1998); and soil organic matter (SOM) was measured as a percent of dry soil following loss on ignition (Combs and Nathan 1998).

In spring of 2013, a switchgrass biomass study was initiated to examine yield response to P. Prior to planting, pelletized lime was broadcast applied to the site at a rate of 2242 kg ENP (Effective Neutralizing Power) ha^{-1}, based on University of Minnesota recommendations (Kaiser et al. 2011a). The lime was not incorporated, however, because the experiment involved seeding into standing oat (*Avena sativa*) stubble, necessitating minimal disturbance to the soil surface. Phosphorus, as triple superphosphate (TSP, 0-45-0), was applied with the seed at planting, below the soil surface, to minimize fixation with lime. P treatments were applied at rates of 0, 9.8, 19.6 and 29.3 kg P ha^{-1}. Atrazine (2-chloro-4-ethylamino-6-isoproplyamino-*s*-triazine) was applied at 2.3 l ha^{-1}, pre-emergence, for weed control. Switchgrass germination was very poor, however, and the stand failed to establish. Standing biomass was killed using glyphosate (*N*-

(phosphonomethyl)glycine) in August, 2013. All plant material was removed in the fall of 2013, followed by shallow (10 cm) field cultivation. Cultivation was aligned in the direction of applied P treatments to minimize cross-plot P movement. After cultivation, composite soil samples (0-15 cm depth) were collected from each treatment plot to establish baseline soil test P values for the current study, initiated in 2014. Samples were submitted to the University of Minnesota Research Analytical Laboratory for testing, as described above, and the resulting soil test P values were low to medium (Table 3-2). The original plot layout and P treatments were maintained, and P fertilizer was reapplied prior to switchgrass establishment. Phosphorus treatments were broadcast applied by hand, as triple superphosphate (TSP, 0-45-0), at rates of 0, 9.8, 19.6 and 29.3 kg P ha^{-1} and incorporated prior to planting. Because P was applied in both 2013 and 2014 with minimal biomass production and removal in 2013, the total P applied in this study was considered to be 0, 19.6, 39.1 and 58.6 kg P ha^{-1}.

The experimental design was a split plot, randomized complete block with four replications. Main plot treatment was switchgrass and subplot treatment was P rate, as described above. The switchgrass cultivars used in this study were ‘Shawnee’, ‘Sunburst’, and ‘Liberty’. ‘Shawnee’ and ‘Sunburst’ are hardy upland forage cultivars, and ‘Liberty’ is the first lowland-type cultivar adapted to USDA plant hardiness zones 4, 5, and 6, bred specifically for bioenergy production, (Vogel et al. 1996; Boe and Ross 1998; Vogel et al. 2014). A further description of each switchgrass cultivar can be found in Chapter 1 (this volume).

Plots were seeded in June 2014, with 11.2 kg seed ha^{-1} using a Wintersteiger Plotmaster small plot grain drill (Wintersteiger Inc., Salt Lake City, Utah). Seed was planted into a firm, smooth seedbed at a depth of 6 to 13 mm in rows 15.2 cm apart. Pre-emergent atrazine was applied at 2.3 L ha^{-1} and 2,4-D amine salt (2,4-dichlorophenoxyacetic acid) was applied at 2.3 L ha^{-1}, as needed for broadleaf weed control, for the duration of the study. No N fertilizer was applied in the establishment year to minimize weed competition. In late spring of the second and third growing seasons, 56 kg N ha^{-1} in the form of urea (46-0-0) coated with urease inhibitor NBPT (N-(n-butyl) thiophosphoric triamide) was broadcast surface applied to all plots.

Stand establishment was evaluated in May 2015, based on the frequency grid of Vogel and Masters (2001): presence or absence of desired species was counted within a randomly-placed metal grid containing 25 squares of 15 cm by 15 cm. The count was completed four times per plot and plant density (plants m^{-2}) was obtained by multiplying the frequency of occurrence by 0.4.

Biomass harvest and analyses

Biomass was harvested annually, following a killing frost, for three years using a Carter plot forage harvester (Carter Manufacturing Company, Inc., Brookston, Indiana) with a cutting height of approximately 10 cm and width of 0.9 m. Plots were cut down the center, avoiding the outer edges to reduce edge effects. Harvested biomass was weighed in the field. Prior to mechanical harvest, two subsamples were hand-cut within each subplot. The subsamples were refrigerated at 10°C until processing, at which point they were

separated into weed and grass components, weighed, dried at 50°C in a forced-air oven for at least 72 hours, and reweighed to determine percent dry matter (DM). Total DM for each sample was calculated using the field harvest wet weight plus subsample wet weight, multiplied by percent DM. After drying, subsamples were ground to pass a 2-mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ) and combined to obtain one sample per plot. Plant tissue analyses were conducted on 2014 and 2015 samples only, using the University of Minnesota's Research and Analytical Laboratory. Total N was determined by combustion, using a LECO Nitrogen Analyzer (LECO Corporation, St. Joseph, Michigan). Total Al, B, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, and Zn, was determined using an ARL (Fisons) Model 3560 Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) (Thermo Instrument Systems Inc. (Fisons Instruments Inc. Division), Waltham, Massachusetts). Total biomass nutrient removal was calculated using nutrient concentration multiplied by biomass DM.

Soil sampling and laboratory analyses

Plant roots were collected near peak biomass production in August of 2014 and 2015 from control (0 kg P ha^{-1}) plots and plots fertilized at 58.6 kg P ha^{-1}. Three plants in each plot were identified for collection and standing grass was trimmed to approximately 2 cm above ground level. Plants were removed to a depth of 15 cm using a Giddings hydraulic probe fitted with a 7 cm diameter bit and steel tube (Giddings Machine Company, Inc., Windsor, Colorado). To minimize contamination between samples, each sample was collected into a 15 cm × 6 cm diameter plastic auger tube liner fitted with

vinyl end caps (Giddings Machine Company, Inc., Windsor, Colorado). The auger bit and steel tube were cleaned with 70% ethanol between samples. Samples were stored on ice immediately after collection and transported back to the laboratory, where they were stored at 20°C while rhizosphere soil extraction was completed (approximately three weeks).

Rhizosphere soil was separated following the protocol of Fernandez et al. (2016). Individual roots were separated from plant crowns and shaken to remove non-adhering soil. Root pieces were then placed into sterile 50mL collection tubes and agitated on a horizontal shaker table for 30 minutes in 35mL of autoclaved 0.1M $(NH_4)_2HPO_4$ with 1% gelatin buffer. Roots were removed and the soil suspension was centrifuged at 7500 × *g* for 20 minutes. After discarding the supernatant, the remaining soil pellet was stored at -80°C.

Gravimetric soil moisture was obtained by weighing approximately 12 g of field-moist soil from each soil core in a tin weigh boat and reweighing after drying at 105°C for a minimum of 24 hours. Soil chemical analyses were conducted by the University of Minnesota Research Analytical Laboratory using soil from each soil core. However, not all analyses were conducted on samples in each year due to unanticipated sample loss (2014) and contamination (2015). In 2014, soil P was determined using the Bray P1 test, and extractable Fe, Mn, Zn, Cu, Pb, Ni, Cd, and Cr were determined in air-dried soil by extraction using 0.005 M DTPA (diethylenetriaminepentaacetic acid) with the resulting filtrate analyzed using an ARL (Fisons) Model 3560 inductively-coupled plasma atomic emission spectrometer (ICP-AES) (Thermo Instrument Systems Inc. (Fisons Instruments

Inc. Division), Waltham, MA) (Fassel and Kniseley 1974; Dahlquist and Knoll 1978; Baker and Amacher 1982; Whitney 1998). In 2015, soil pH was evaluated as described for pre-plant soil analyses. Soil NO_3-N and NH_4-N were evaluated on air-dried samples as follows: NO_3-N was extracted with 0.01 M $CaSO_4$ and NH_4-N was extracted with 2 M KCl, with the resulting filtrate from each test measured on a Lachat Quikchem 8500 Flow Injection Analyzer (Hatch Company, Loveland, CO) (Henricksen and Selmer-Olsen 1970; Keeney and Nelson 1982; Willis and Gentry 1987; Gelderman and Beagle 1998). Soil samples (0-15 cm depth) were also collected from control plots and plots fertilized at 58.6 kg P ha^{-1} at the conclusion of the study (spring 2017) and evaluated for soil P using the Bray P1 test.

Root biomass determination

Because soil core volume was consistent between samples, root biomass was used as a proxy for root density within each sample. Root biomass was determined by using a Fine Root Extraction Device (FRED) (Pallant et al. 1993), modified by Vargas et al. (personal communication, 2015), to separate soil and debris from roots using gentle agitation with bubbles in water. A full description of the modified FRED and root extraction protocol is available in Chapter 2, this volume. Soil core samples were prepared the day prior to extraction: each sample was placed into a jar filled with a 5% (w/v) solution of sodium hexametaphosphate to facilitate clay dispersion, after Marriott and Wander (2006). Each sample was agitated on a horizontal shaking table for 30 minutes

prior to root extraction in the FRED. Following extraction, all roots were dried in a 60°C oven for a minimum of 48 hours and weighed to determine biomass.

Next-generation Illumina sequencing

DNA was extracted from each rhizosphere soil sample using MoBio PowerSoil kits (MoBio Laboratories, Carlsbad, CA). Amplicon preparation and sequencing was performed by the University of Minnesota Genomics Center (UMGC, Minneapolis, MN). Bacterial sequencing was performed using the BSF784/R1064 primer set (Claesson et al. 2010) targeting the V5V6 hypervariable regions of the 16S rRNA gene. Fungal sequencing was performed using the ITS1F primer targeting the ITS1 region of the rRNA gene. Illumina (San Diego, CA, USA) sequencing adapters and indices were then added by UMGC using the dual index method (Gohl et al. 2016) for bacteria and the Earth Microbiome Project (EMP) protocol for fungi (http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/its/). Sterile water negative controls were carried through amplification and sequencing. The 2014 16S and all fungal sequencing was performed on the Illumina HiSeq 2500 platform, and 2015 16S sequencing was performed on the MiSeq platform; comparable results have been shown across platforms (Caporaso et al. 2012).

Sequence processing and analysis

All sequence data were processed and analyzed using mothur v. 1.35.1 (Schloss et al. 2009). The 16S sequence data were trimmed to the first 150 nucleotides (nt), paired-

end joined using fastq-join (Aronesty 2013), and screened to meet the following quality criteria: no ambiguous bases (N), homopolymer length ≥ 8 nt, average quality score ≥ 35 over a window of 50 nt, ≤ 1 differences to a primer sequence, and identical to a barcode sequence. Chimeras were removed using UCHIME (Edgar et al. 2011), and sequences were aligned to the SILVA database, ver. 123 (Quast et al. 2013). For statistical comparisons, sequence numbers per sample were normalized, by random subsampling, to 11,000 reads per sample, and samples not meeting this criteria were removed from further processing. Sequences were clustered into operational taxonomic units (OTUs), assigned at 97% similarity using the furthest neighbor algorithm, and classified against the Ribosomal Database Project ver. 14 (Cole et al. 2009). ITS1 sequence data were trimmed to the first 250 nt and processed similarly to the 16S sequences and only forward-read sequences were used. Sequences displaying any differences to a primer were removed. Fungal sequences were aligned to the UNITE database ver. 6 (Koljalg et al. 2014). Samples were normalized by subsampling to 20,000 reads per sample, and OTUs were also classified against the UNITE database. Sequence data were deposited to the Sequence Read Archive of the National Center for Biotechnology Information under accession number PRJNA387437.

Statistical analysis

Biomass and P removal data were analyzed using mixed linear models (PROC MIXED) in the SAS software program, Version 9.4 of the SAS System for Windows (Copyright © 2002-2012, SAS Institute Inc., Cary, North Carolina). We used a split-split plot

design with cultivar as the whole plot, P rate as the subplot, and year as the sub-subplot (Steel et al. 1997). The P rate treatments were separated into linear and quadratic components using orthogonal contrasts. All datasets met analysis of variance normality assumptions. Replication was treated as a random effect; all other effects were treated as fixed (Appendix B). Significant differences, based on $\alpha = 0.05$, were determined using 'lsmeans' with the 'pdiff' option. Regression equations and statistics were calculated using PROC REG. Analysis of root biomass and soil P for each sample were analyzed using one-way analysis of variance in PROC GLM in the SAS software program. Each plant and its associated soil P were treated as individual subsamples and were incorporated into the analysis of variance through isolation of the subsampling error within the model, thereby reducing the total experimental error variance (Steel et al. 1997, p.223). Correct fixed-effect tests for significance were specified using 'test' statements.

Unless otherwise indicated, bioinformatics statistics were performed using mothur ver. 1.35.1. (Schloss et al. 2009). Number of OTUs (S_{obs}), Good's coverage, Shannon index, and abundance-based coverage estimate (ACE) were calculated for each sample. Analysis of variance on richness and diversity indices as a function of treatment were performed using PROC GLM as described for root biomass, above. Bray-Curtis distance matrices were calculated for community comparisons (Bray and Curtis 1957), including principal coordinate analyses (PCoA), analysis of similarity (ANOSIM) (Clarke 1993) and analysis of molecular variance (AMOVA) (Excoffier et al. 1992). Replicates were grouped by treatment for ANOSIM and AMOVA analyses using .design files. Linear discriminant analysis (LDA), used to determine significant differences in taxa as a

function of treatment, was performed using the LDA Effect Size (LEfSe) tool (Segata et al. 2011) in Galaxy (Afgan et al. 2016). To assess and visualize soil/plant physiochemical associations with bacterial orders, a redundancy analysis (RDA) was prepared in R-studio (v 1.0.34) (R Core Team 2016) utilizing the vegan package. Variance partitioning was performed in R-studio using constrained RDA as described by Borcard et al. (1992). Spearman correlation values associated with the RDA were generated using XLSTAT Ecology (v 18.07) (Addinsoft 2017). Statistical analyses were evaluated based on $\alpha = 0.05$, except where a Bonferroni correction was applied to minimize multiple comparison errors in pairwise ANOSIM, AMOVA, and Spearman correlations.

Results

Biomass yield and phosphorus removal

Biomass yield was influenced by the main effect of P fertilization rate ($p = 0.046$) and the year × cultivar interaction ($p = 0.044$) (Table 3-3). Biomass yield increased linearly in response to increasing P application (Figure 3-1). Unfertilized yields averaged 9.5 Mg ha^{-1} yr^{-1}, less than yields fertilized at 39.1 and 58.6 kg P ha^{-1}, which averaged 10.3 Mg ha^{-1} yr^{-1} ($p < 0.05$). The year × cultivar interaction resulted from a difference in magnitude of 'Liberty' biomass yields in 2015 relative to other year and cultivar combinations. However, there were no differences in ranking or direction among cultivars. Therefore, to simplify the results, we will discuss only main effects of cultivar ($p < 0.001$) and year ($p < 0.001$). 'Shawnee' produced greater biomass than 'Liberty', and both produced greater biomass than 'Sunburst', averaging 11.3, 10.2, and 8.5 Mg ha^{-1} yr^{-1},

respectively. Annual yields increased every year; yields from 2014-2016 averaged 7.7, 9.6, and 12.8 Mg ha^{-1} yr^{-1}, respectively.

Phosphorus tissue concentration and removal was also affected by the cultivar × year interaction ($p < 0.001$ and $p = 0.003$, respectively) (Table 3-4). In 2014, 'Sunburst' tissue P concentration was greater than any other year and cultivar combination, resulting in greater P removal relative to all but 2015 'Shawnee' biomass. 2015 'Shawnee', however, had nearly 30% greater yield than any other cultivar in 2014 or 2015, corresponding to greater P removal. Phosphorus removal in 2015 'Liberty' biomass was less than all other year and cultivar combinations, largely as a result of lesser P tissue concentration and yield that was adversely impacted by weed biomass. Phosphorus tissue concentration and removal were also affected by P rate ($p = 0.007$ and $p < 0.001$, respectively), with both increasing linearly in response to increasing P application. Phosphorus tissue concentration and removal at 0 and 19.6 kg P ha^{-1} were similar, averaging 718 mg P kg^{-1} biomass and 5.6 kg P removed ha^{-1} yr^{-1}, respectively. Phosphorus tissue concentration and removal at 39.1 and 58.6 kg P ha^{-1} were also similar, averaging 807.8 mg P kg^{-1} biomass and 6.9 kg P removed ha^{-1} yr^{-1}, respectively. At the conclusion of the study, soil test P in unfertilized plots was less than soil test P in plots fertilized at 58.6 kg P ha^{-1}, averaging 8.25 ± 2.3 and 15.6 ± 2.6 mg kg^{-1}, respectively ($p < 0.001$).

Root biomass was not affected by cultivar ($p = 0.599$) or P fertilization ($p = 0.126$). However, root biomass was greater in 2015 than in 2014 ($p < 0.001$), averaging 3.0 ± 1.0 and 1.6 ± 0.6 g, respectively.

Alpha diversity and composition of bacterial community

A total of 134 samples were analyzed, including 72 samples from 2014 and 62 samples from 2015, with mean Good's coverage of 88.0 ± 1.6%. A total of 31,435 operational taxonomic units (OTUs) were identified from all samples, with 23,696 OTUs in 2014 and 21,000 OTUs in 2015. Bacterial diversity (Shannon index) and richness (abundance weighted coverage) were not affected by cultivar or P fertilization treatment, and only richness differed as a function of year ($p < 0.001$, Table 3-5).

OTUs were classified into 166 bacterial orders in 2014 and 170 orders in 2015, with 6.5% and 6.2% of reads unclassified at the order level in 2014 and 2015, respectively. The most abundant 12 orders in each year, separated by cultivar, are shown in Figure 3-2. In 2014, the most abundant bacterial orders in both 'Liberty' and 'Shawnee' were *Burkholderiales* (12.9 and 10.4% respectively) followed by *Actinomycetales* (10.0 and 9.8%, respectively) and unclassified orders. In 'Sunburst' the most abundant order was *Actinomycetales* (11.2%) followed by *Burkholderiales* (9.5%). In 2015, the most abundant bacterial order in all cultivars was *Actinomycetales*, with 12.3, 12.1 and 11.3% relative abundance in 'Liberty', 'Shawnee', and 'Sunburst' respectively. *Burkholderiales* was second-most abundant in 'Liberty' (7.5%) and 'Shawnee' (6.7%), while unclassified orders were second-most abundant in 'Sunburst' (6.0%).

Factors affecting bacterial community composition

Community composition differed as a result of the interaction between cultivar, P application rate, and year ($p < 0.001$), but the taxonomic diversity was too great to resolve

discrete patterns related to the abundance of orders in pair-wise comparisons. Therefore, the evaluation of treatment effects on bacterial diversity was conducted in two steps: First, to determine whether there was a persistent cultivar and P fertilization influence on beta diversity through time, all samples from both years were analyzed together. Then, samples were split by year, and main effects of cultivar and P fertilization on beta diversity were examined separately within each year.

When samples from both years were combined, ANOSIM revealed no differences in beta diversity as a function of P fertilizer application ($p = 0.587$), despite greater soil P concentrations in fertilized (22.3 ± 7.7 mg kg^{-1}) as compared to unfertilized (13.4 ± 3.8 mg kg^{-1}) soil core samples ($p < 0.001$ in 2014, soil P unavailable in 2015). Cultivar influenced community composition, however ($p < 0.001$). Linear discriminant analysis revealed that two bacterial orders, *Burkholderiales* and *Acidobacteria Gp. 1 Terriglobus*, differed by cultivar, and both were more abundant in 'Liberty' than in any other cultivar ($p < 0.05$). Results from redundancy analysis (RDA), used to partition variance in community structure also indicated that the bacterial community structure was most strongly associated with cultivar differences rather than P application rate. For example, 92.6% of the variation in community structure, as a function of relative abundance, was explainable by cultivar, whereas 7.2% of the variation was explained by P rate, and less than 1.0% of the variation was explainable by a combination of both cultivar and P fertilization effects.

Ordination of Bray-Curtis dissimilarity distances by principal coordinate analysis and AMOVA ($p <0.001$) revealed clear clustering of samples by year (Figure 3-3). There were 82 bacterial orders that differed between years ($p < 0.05$, data not shown), including

several of the most abundant orders. *Burkholderiales*, *Acidobacteria Gp. 6*, and *Sphingomonadales* were more abundant in 2014, while *Pseudomonadales*, *Actinomycetales*, and *Rhizobiales* were more abundant in 2015.

In 2014 samples, beta diversity was not affected by the cultivar × P fertilization rate interaction ($p = 0.077$), nor was it influenced by P fertilization rate ($p = 0.931$). While there were overall differences in community composition as a function of cultivar ($p = 0.009$), linear discriminant analysis revealed no bacterial orders that were consistently different among cultivars. In 2015 samples, the cultivar × P rate interaction did influence beta diversity ($p=0.011$), although no pairwise comparisons were significant, indicating that there were no bacterial orders that were consistently discernable across replicates on a pairwise basis. Similar to 2014, the main effect of P fertilization rate did not contribute to community differences ($p = 0.526$), but cultivar did ($p = 0.001$). Linear discriminant analysis indicated that one bacterial order, *Actinobacteria Acidimicrobiales*, was more abundant in the 'Sunburst' rhizosphere than in any other cultivar.

Principal coordinate analysis also revealed clustering of samples as a function of differences in spatial variability of soil physiochemical characteristics, as determined by samples located within each experimental block (AMOVA $p <0.001$, both when averaged over years as well as for 2014 and 2015, separately). Redundancy analysis supports these results, indicating that 63.1% of the variation in community structure was explainable by experimental block, whereas 35.5% of the variation was explainable by the combination of cultivar and P fertilization treatments, and only 1.4% of the variation was explainable by a combination of both block and treatment effects.

Plant and soil characteristics affecting bacterial community composition

Redundancy analysis indicated that the bacterial community structure was more strongly associated with plant and soil parameters than with P fertilization or cultivar treatments in each year, although the observed soil and plant parameters were not identical between years. In 2014, 87.7% of the variation in community structure was explainable by the combination of plant/soil parameters, while only 5.3% was explainable by treatment effects of cultivar and N, and 7.0% of the variance was explainable by a combination of both treatment effects and plant/soil parameters (Figure 3-4A). Among the most strongly influenced bacterial orders in 2014 (Figure 3-5A), there were no correlations with biomass yield, root biomass, or soil moisture (Table 3-6). Soil P was positively correlated with increasing abundance of *Acidiobacteria Gp. 4* and *Acidobacteria Gp. 6*, although plant tissue P concentration was not correlated with any bacterial order. *Burkholderiales* abundance was not correlated with any soil parameters. *Actinomycetales* was negatively correlated with several soil metals (Fe, Zn, Pb, Ni, Cd, Cr). Soil Fe, Zn, Cd, and Cr concentrations were among the most influential soil chemical parameters in 2014. Some orders were similarly correlated with the same element concentration in both plant tissue and soil, such as *Actinomycetales* negatively correlated with Pb concentration. While plant Mg, Ni, and Pb concentrations were correlated with all orders other than *Burkholderiales* most other plant parameters were not correlated with abundance of any bacterial orders. There was no correlation between labile soil P and soil Pb ($p = 0.062$).

In 2015, plant/soil parameters accounted for 86.5% of the total explainable variance, treatment effects accounted for 9.0%, and joint plant/soil and treatment effects

explained 4.5% of the total variance (Figure 3-4B). In 2015, abundance of *Acidobacteria Gp. 4* was positively correlated with soil moisture, but no other orders were correlated with soil moisture, biomass yield, root biomass, soil NH_4^+-N or soil NO_3^--N (Figure 3-5B, Table 3-7). Soil pH was positively correlated with abundance of *Acidobacteria Gp. 4*, but negatively correlated with abundance of *Acidobacteria Gp. 6*. *Actinomycetales* and *Pseudomonadales* were not correlated with any soil or plant parameter, and similar to 2014, most plant parameters were not correlated with abundance of any bacterial orders. The only similarity between 2014 and 2015 was the negative correlation between *Acidiobacteria Gp. 1* and plant Pb concentration.

Alpha diversity and composition of fungal community

A total of 143 samples were analyzed, including 71 samples from 2014 and 72 samples from 2015, with mean Good's coverage of 99.9% ± 0.0%. A total of 1,602 OTUs were identified from all samples, but 200 OTUs, encompassing 73.8% of sequence reads, were unable to be classified at any taxonomic level. There were 946 OTUs identified in 2014 and 1,170 OTUs identified in 2015. Shannon index averaged 1.43 ± 0.15 in 2014 and 1.49 ± 0.17 in 2015 ($p = 0.142$). Community richness averaged 134 ± 38 in 2014 and 150 ± 42 in 2015 ($p = 0.009$). As in the bacterial community, neither diversity nor richness in the fungal community was influenced by cultivar or P fertilizer treatments.

In 2014, there were 552 OTUs classified to the species level, including 189 singleton species (only one representative of that species was found in any sample), with 73.9% of sequence reads unable to be classified at the species level. In 2015, 630 OTUs

were classified to the species level, including 224 singletons, with 73.8% of sequence reads unclassified at the species level. The 12 most abundant fungal OTUs, separated by year and cultivar, are shown in Figure 3-6. None of the most abundant species were AM fungi, *and less than 0.01% of sequence reads were classified to Glomeromycota*.

Factors affecting fungal community composition

Patterns of beta diversity differences among fungal communities were similar to those in the bacterial community. The interaction between cultivar, P application rate, and year influenced fungal beta diversity ($p < 0.001$), although linear discriminate analysis could not resolve discrete pairwise differences for any particular species. As with the bacterial community, fungal community composition was first analyzed to explore a persistent cultivar and P fertilization influence on beta diversity through time, with all samples from both years analyzed together. Then, main effects of cultivar and P fertilization on beta diversity were then examined within each year individually.

When averaged over years, there was a significant main effect of cultivar ($p < 0.001$), but not of P fertilization ($p = 0.668$). *Fusarium sp. BS 8* was more abundant in the 'Sunburst' rhizosphere, while *Fusicolla violacea* was more abundant in the 'Liberty' rhizosphere. Variance partitioning by RDA indicated that 56.8% of the variation in fungal community structure was attributable to cultivar, 43.4% was attributable to P fertilization, and -0.2% was attributable to the combined effect of cultivar and P fertilization. Negative variance components are possible in redundancy analyses of ecological data (Borcard et al. 1992) and can arise from particularly complex relationships between predictor

variables (Okland 2003) and/or as a result of external factors other than the predictor variables used in the model (Qioghong and Bratkenhielm 1995). However, the large proportion of variance explained by P fertilization did not align with ANOSIM results for beta diversity. It is possible that either external factors, the existence of a majority 'unclassified' species (74%), or a large percentage of singleton OTUs (34%) influenced the negative variance. After removing the 'unclassified' species category, RDA results better reflected ANOSIM results and were similar to those from the bacterial community analysis: 77.1% of the variation in fungal community composition was explainable by cultivar, 23.0% was explainable by P fertilization, and the joint effect of cultivar and P received a variance component of – 0.01%.

Principal coordinate analysis and AMOVA ($p < 0.001$) revealed clustering of samples by year (Figure 3-7), but clustering was less clear than for bacterial communities. Linear discriminant analysis revealed 38 fungal species that were different between years ($p < 0.05$, data not shown), including some of the most abundant species. *Cryptococcus terreus*, *Fusarium fujikuroi*, and *Phallus rugulosus* were more abundant in 2014, while *Atheliacea*, *Ascomycota sp. FL 2010c*, and *Fusicolla violacea* were more abundant in 2015. There were no differences in species belonging to *Glomeromycota*.

In 2014, cultivar and fertility treatments did not influence community structure: $p = 0.054$, 0.780, and 0.092 for cultivar × P fertilization interaction, P fertilization main effect, and cultivar main effect, respectively. In 2015, the cultivar × P rate interaction was significant ($p < 0.001$). Differences occurred between 'Liberty' and 'Sunburst' ($p = 0.001$) and 'Shawnee' and 'Sunburst' ($p = 0.003$) under unfertilized P conditions, and between

unfertilized P ‘Sunburst’ and fertilized ‘Liberty’ (p=0.003), although linear discriminate analysis could not resolve consistent differences of any particular species among cultivars. Therefore, we examined main effects of P fertilization ($p = 0.870$), and cultivar ($p < 0.001$). Linear discriminant analysis revealed the same differences by cultivar in 2015 as when averaged over 2014 and 2015: *Fusarium sp. BS 8* was more abundant in the ‘Sunburst’ rhizosphere, while *Fusicolla violacea* was more abundant in the ‘Liberty’ rhizosphere.

Similar to the bacterial community analysis, there was a spatial component (block) effect on community composition (ANOSIM <0.001) relative to treatment effects. With ‘unclassified’ species removed, RDA results indicated that a slight majority of the variation in community structure was explainable by block, 52.2%, with 47.8% explainable by cultivar and P fertilization treatment. There was a slight negative variance component (-0.4%) associated with the combined effects of treatment and block.

Plant and soil characteristics affecting fungal community composition

Similar to results from the bacterial community, the fungal community structure was more strongly associated with plant and soil parameters than with P fertilization or cultivar treatments in both years. In 2014, 95.1% of the variation in community structure was explainable by the combination of plant/soil parameters. In contrast, only 6.0% was explainable by treatment effects of cultivar and N, the combination of both treatment effects and plant/soil parameters had a slight negative variation component at -1.1% (Figure 3-8A). Among the most strongly influenced fungal species in 2014 (Figure 3-9A),

there were no correlations with biomass yield, root biomass, soil moisture, or soil P (Table 3-8). In addition, *Fusarium spp.* were not correlated with any soil or plant parameters. No species were similarly correlated with the same element concentration in soil and plant tissue. In 2015, plant/soil parameters accounted for 82.7% of the total explainable variance, treatment effects accounted for 8.7%, and joint plant/soil and treatment effects explained 8.6% of the total variance (Figure 3-8B). Among the most strongly influenced fungal species in 2015 (Figure 3-9B), there were no correlations with root biomass, and no correlations between plant/soil parameters and *Ascomycota sp. FL 2010c* or *Ramaria coulterae* (Table 3-9). *Atheliaceae sp.* was positively correlated with biomass yield and negatively correlated with soil NO_3^-. There were no similarly correlated species between 2014 and 2015.

Discussion

Biomass yield results indicated that 'Shawnee' was more productive relative to 'Sunburst' or 'Liberty', when averaging over year and P fertilization rate. These results diverge somewhat from a switchgrass cultivar and N rate study located less than 5 km away, which found that total post-frost yield of 'Shawnee' and 'Sunburst' were similar over a period of three years, and both produced greater biomass than did 'Liberty' (Chapter 1, this volume). That study, however, was initiated one year prior to the current study, did not include establishment year biomass, and was conducted on an eroded loam soil. Results from both of these studies affirm that differences in switchgrass biomass yield as a function of cultivar, soil characteristics, and climate conditions are to be

expected (Casler and Boe 2003; Vogel et al. 2014), even when grown under similar circumstances, although 'Shawnee' may be more consistently productive than either 'Sunburst' or 'Liberty'.

Biomass yield results also indicated that supplying P fertilizer at a rate of between 19.6 and 39.1 kg P ha^{-1}, in conjunction with optimum N fertilizer (Chapter 1, this volume) can increase production relative to the control when soil test P is in the medium range. The linear yield response to P was similar to that described by Kering et al. (2012), whose three-year study of switchgrass biomass yield as a function of P application rate on low soil test P soils in Oklahoma found a positive linear response in post-frost biomass production with increasing P fertilizer at one of two locations. Others have found limited (Morris et al. 1982) or no (Hall et al. 1982) yield response to P fertilizer on low P test soils, while some have found a yield response to P in the context of a significant N x P interaction in low soil test P soils (Rehm 1984; Rehm 1990).

Phosphorus tissue concentration differed as a function of cultivar, similar to results from Morris et al. (1982). Our overall average annual P removal was 6.3 ± 1.8 kg P ha^{-1} yr^{-1}, well within the range of Jungers et al. (2015b), who found P removal to average 8 ± 1 kg P ha^{-1} yr^{-1} over two years in mature (3-4 year) 'Sunburst' switchgrass grown on a very high P test soil and fertilized at 56 kg N ha^{-1} annually. Maximum annual P removal in this study was in 2014 'Sunburst' biomass, which averaged 7.8 ± 2.3 kg P ha^{-1}. While not all added P fertilizer is plant-available, an application rate of 39.1 kg P ha^{-1} may take 2-3 years before biomass harvest removes an amount equivalent to applied P. Soil test P will decrease over time and should be monitored accordingly to maintain stand productivity.

While the symbiotic relationships formed between switchgrass and AM fungi can confound results from P rate studies (Hetrick et al. 1990; Brejda et al. 1993), our survey of the fungal community did not identify a robust AM fungal population in the rhizosphere, despite visual observations of fungal hyphae in association with plant roots during sample preparation. Fungal community composition results should be interpreted with caution, given known issues associated with fungal taxonomy classification and next-generation sequencing of fungal communities (Lindahl et al. 2013; Nguyen et al. 2015; Staley et al. 2017). For example, less than 2% of the estimated millions of global fungal taxa have been formally described, and shortcomings in primer attributes and sequencing methods do not equally amplify all fungal lineages nor do they provide robust estimates of total species richness and abundance (Taylor et al. 2016). The AM fungal phylum, *Glomeromycota*, is particularly challenging for primer selection and amplification, given the lack of taxonomic information associated with the phylum (Lindahl et al. 2013).

Bacterial and fungal community diversity and richness were not affected by treatment, but community richness differed from year to year in both communities. A change in rhizosphere community richness over time is not unexpected; plants affect changes by selecting for specific microbial populations from the community of natural inoculum in the soil (Berg and Smalla 2009; Bulgarelli et al. 2012). Changes in community composition over time are well documented, largely in response to changes in root morphology, root exudate quantity and composition, and soil physiochemical status (e.g. labile C, pH, moisture) (e.g. da Rocha et al. 2009; Philippot et al. 2013; Mao et al. 2014b; Hargreaves et al. 2015). However, we found no correlations between bacterial or

fungal taxa and root biomass in either year, similar to results from Chapter 2 (this volume).

The most abundant bacterial orders found in this study, including *Actinomycetetales*, *Acidobacteria spp.* and *Burkholderiales*, belonging to the *Actinobacteria*, *Adicotacteria*, and *Proteobacteria* phyla, respectively, have been shown to be among the most abundant bacterial phyla in the switchgrass rhizosphere in other studies (Mao et al. 2014b; Rodrigues et al. 2016). *Burkholderiales*, in particular, have been shown to actively utilize root exudates from switchgrass (Mao et al. 2014b). Among the most abundant fungal species, several are known plant pathogens, e.g. *Fusarium* spp. and *Rhizoctonia zeae* (Gwinn and Gavin 1992; Sneh et al. 1996; Geiser et al. 2013). Others include saprotrophs, e.g. *Phallus sp.* (Trierveiler-Pereira et al. 2014) and yeasts, *Cryptococcus terreus* and *Cryptococcus terricola*, which were also found to be among the most abundant fungal species in the rhizosphere of strawberry (*Fragaria × ananassa)* (Nallanchakravarthula et al. 2014). However, none of the most abundant fungal species were AM fungi, similar to results from Mao et al. (2014a), who used 18s rRNA gene amplification and sequencing to identify rhizosphere eukaryotic communities. Given that 74% of fungal OTUs were unclassified, further exploration of the switchgrass fungal community may only be possible with improved sequencing and bioinformatics approaches as well as improvement in fungal taxonomic databases (Staley et al. 2017).

Similar to results from other studies (Knox et al. 2014; Winston et al. 2014; Rodrigues et al. 2016; Wen et al. 2017), we found that switchgrass cultivar influences the composition of the rhizosphere microbial community. Very few bacterial orders or fungal

species, however, differed consistently among cultivars across replicates. This result is not unexpected, given the profound variability of microbial taxa in soil; even within a single species of bacteria, the composition and abundance of strains can vary significantly between individual rhizospheres of the same plant species in the same field (Ramette et al. 2005). Furthermore, the functional traits in bacterial communities have been shown to be conserved - in spite of significant differences in diversity - as a result of functional redundancy among bacteria (Lozupone et al. 2012).

Phosphorus fertilization did not influence rhizosphere community composition, despite differences in soil P levels among treatments. Had there been an influence of soil P among bacterial communities, we may have anticipated differences in *Pseudomoas*, *Bacillus*, and *Rhizobium* bacterial genera, which are known to include P-solubilizing strains (Rodríguez and Fraga 1999). The lack of P fertilization effects on fungal communities are likely a result of issues with fungal sequencing, analyses, and taxonomy identification, as previously described.

The majority of the variation in both bacterial and fungal community structure, taken as relative abundances of orders, was largely explainable by the combination of plant/soil parameters rather than treatment effects. These results suggest that the existing soil microbial community, shaped by antecedent soil and climate characteristics, had a larger effect on microbial community composition than did plant rhizospheres. These results are consistent with those from many other studies (e.g. Bulgarelli et al. 2012; Bakker et al. 2013; Nallanchakravarthula et al. 2014; Hargreaves et al. 2015).

Finally, we also found that both bacterial and fungal community structure were different as a function of location within plot, when evaluated by block. This may be a result of factors such as organic matter content, nutrient status, pH, moisture or other factors that contribute to the extreme microbial diversity in soil and complicate attempts to characterize the microbial community (Baker et al. 2009; Schmidt and Waldron 2015). While microbial community diversity can vary greatly from soil aggregate to soil aggregate within the same soil sample (Blackwood et al. 2006), samples collected in close proximity to one another have been shown to have less variability in community composition than between samples collected at a larger scale (Baker et al. 2009), similar to our results. Furthermore, the indeterminate growth habits and considerable spatial extent of fungal mycelia can result in repeated sampling of the same individual over a distance of several meters (Lindahl et al. 2013).

Conclusions

In this experiment, 'Shawnee' switchgrass, an upland forage variety, provided optimum post-frost biomass yield when fertilized with at least 19.6 kg P ha^{-1}, applied in split application prior to establishment, on a low to medium soil P test clay loam soil. Although more research is necessary to make recommendations for P application under similar circumstances, a target application rate is likely between 19.6 and 39.1 kg P ha^{-1}. We also found that 'Liberty' did not produce as much biomass as did 'Shawnee', although both were more productive than 'Sunburst'. Results also indicated that soil and plant characteristics had a stronger influence on shaping the rhizosphere microbiome than did

treatment effects, although cultivar also affected the community composition of rhizosphere bacteria and fungi in switchgrass. Phosphorus fertilization, however, did not influence the community structure, despite a well-documented relationship between switchgrass and AM fungi, which can be of particular importance in low pH or low P soils. Future advances in fungal sequencing, bioinformatics and taxonomy identification may allow for greater insight into fungal community dynamics in switchgrass, leading to more efficient use of P fertilizer for biomass production.

Table 3-1. Monthly precipitation, temperature, and 30-year (1981-2010) averages at Lamberton, MN. 'Sum' refers to annual total precipitation; 'mean' refers to mean annual temperature. Source: Midwestern Regional Climate Center, cli-MATE (MRCC Application Tools Environment, http://mrcc.isws.illinois.edu/CLIMATE/, accessed 4/2017).

Month	30-year average	Year			30-year average	Year		
		2014	2015	2016		2014	2015	2016
	mm				°C			
January	16	18	11	8	-9.6	-12.7	-7.3	-9.2
February	16	13	5	18	-6.9	-12.6	-11.7	-4.4
March	39	25	10	51	-0.5	-4.1	1.1	3.7
April	73	87	31	85	7.5	5.6	8.6	8.6
May	86	46	139	141	14.6	13.8	13.9	14.7
June	103	188	128	66	20.1	20.1	20.3	21.4
July	99	30	96	176	22.1	20.5	21.7	22.1
August	92	94	113	135	20.5	21.0	19.7	21.4
September	86	154	87	134	15.8	16.3	19.5	17.7
October	52	12	41	72	8.6	9.6	10.3	10.1
November	31	13	84	47	-0.3	-4.5	4.1	5.4
December	20	25	34	29	-7.5	-4.8	-2.8	-8.2
Sum/mean	714	705	780	960	7.0	5.7	8.1	8.6

Table 3-2. Soil test results at Lamberton, MN. 2012 test results represent average conditions at the site; 2013 test results represent an average value for each treatment level (± SD). Composite samples from each subplot were tested individually in 2013. Treatment is total P applied over the course of the study.

Year	Treatment	Bray P	Extractable K	Organic matter	Water pH
	kg P ha^{-1}	mg kg^{-1}		%	
2012	N/A	9.0	150.0	4.4	4.9
2013	0	11.3 ± 2.2	187 ± 27	4.2 ± 0.2	5.9 ± 0.2
	19.6	12.2 ± 3.4	190 ± 30	4.2 ± 0.2	5.9 ± 0.2
	39.1	14.0 ± 3.2	196 ± 33	4.3 ± 0.2	5.9 ± 0.2
	58.6	13.7 ± 3.4	188 ± 30	4.2 ± 0.2	5.9 ± 0.2

Table 3-3. Analysis of variance for switchgrass biomass yields (2014-2016) and P removal (2014-2015) as a function of three cultivars and four P rates.

Source of variation	Yield			P removal		
	df	*F* statistic	*P* value	df	*F* statistic	*P* value
Cultivar (C)	2	29.6	<0.001	2	9.7	0.013
Phosphorus (P)	3	3.0	0.046	3	9.7	<0.001
P linear	(1)	7.5	0.011	(1)	23.0	<0.001
P quadratic	(1)	1.6	0.221	(1)	0.9	0.361
C x P	6	0.9	0.523	6	0.1	0.992
Year (Y)	2	111.0	<0.001	1	5.4	0.025
C x Y	4	2.6	0.044	2	6.7	0.003
P x Y	6	0.2	0.967	3	0.6	0.611
C x P x Y	12	0.3	0.995	6	0.2	0.989

Table 3-4. Cultivar × year interaction for tissue P concentration and P removal in switchgrass biomass at Lamberton, MN. Values sharing the same letter are not differ significantly at $\alpha = 0.05$

Year	Cultivar	P concentration	P removal
		mg kg^{-1}	kg ha^{-1}
2014	Liberty	710 b	5.9 b
	Shawnee	750 b	6.2 b
	Sunburst	1247 a	7.8 a
2015	Liberty	495 c	4.5 c
	Shawnee	621 bc	7.1 ab
	Sunburst	753 b	6.2 b

Table 3-5. Year effects on Good's coverage, number of OTUs (S_{obs}), alpha diversity (Shannon Index), and community richness (Abundance Weighted Coverage, or ACE) in bacterial and fungal communities from switchgrass rhizosphere soil. Within each community, significant differences are indicated by letter ($\alpha = 0.05$).

Community	Year	n	Coverage (%)	S_{obs}	Shannon	ACE
Bacterial	2014	72	86.9 ± 1.1	2517 ± 167	6.69 ± 0.17	8120 ± 907 a
	2015	62	89.3 ± 1.0	2284 ± 158	6.62 ± 0.21	5617 ± 676 b
Fungal	2014	71	99.9 ± 0.0	71.8 ± 9	1.44 ± 0.15	134 ± 38 b
	2015	72	99.9 ± 0.0	82 ± 9	1.49 ± 0.17	150 ± 43 a

Table 3-6. Spearman correlation coefficients (r) for plant and soil parameters describing relative abundance of major bacterial orders in 2014. *P* values are shown in parenthesis and significant values, based on Bonferroni adjustment for multiple comparisons, are bolded.

Plant / soil parameters	*Actinomycetales*	*Burkholderiales*	*Spartobacteria* (Unclassified)	*Acidobacteria Gp. 4*	*Planctomycetales*	*Acidobacteria Gp. 6*	*Acidobacteria Gp. 1*
Biomass yield	-0.226 (0.056)	0.191 (0.108)	0.021 (0.863)	-0.018 (0.882)	0.157 (0.186)	-0.117 (0.327)	0.106 (0.373)
Root biomass	0.183 (0.123)	-0.234 (0.048)	-0.074 (0.536)	0.247 (0.036)	-0.181 (0.127)	0.225 (0.057)	-0.117 (0.325)
Soil moisture	-0.342 (0.003)	-0.134 (0.260)	0.341 (0.003)	0.141 (0.237)	0.337 (0.003)	0.185 (0.119)	-0.145 (0.222)
Soil P	-0.212 (0.074)	-0.218 (0.066)	0.213 (0.072)	**0.382 (0.001)**	0.222 (0.061)	**0.402 (<0.001)**	-0.301 (0.010)
Soil Fe	**-0.555 (<0.001)**	-0.167 (0.160)	**0.488 (<0.001)**	**0.502 (<0.001)**	**0.573 (<0.001)**	**0.435 (<0.001)**	-0.282 (0.016)
Soil Mn	-0.111 (0.350)	0.351 (0.002)	-0.011 (0.924)	-0.172 (0.147)	0.117 (0.328)	-0.18 (0.130)	0.164 (0.167)
Soil Zn	**-0.43 (0.000)**	-0.356 (0.002)	**0.471 (<0.001)**	**0.613 (<0.001)**	**0.421 (<0.001)**	**0.634 (<0.001)**	**-0.568 (<0.001)**
Soil Cu	-0.283 (0.016)	0.125 (0.294)	0.311 (0.007)	0.181 (0.128)	0.263 (0.025)	0.145 (0.223)	-0.317 (0.006)
Soil Pb	**-0.382 (0.001)**	-0.031 (0.793)	0.316 (0.007)	0.336 (0.004)	**0.421 (<0.001)**	0.264 (0.025)	-0.193 (0.104)
Soil Ni	**-0.484 (<0.001)**	0.08 (0.504)	**0.428 (<0.001)**	0.258 (0.028)	**0.464 (<0.001)**	0.225 (0.057)	-0.269 (0.022)
Soil Cd	**-0.452 (<0.001)**	-0.252 (0.032)	**0.529 (<0.001)**	**0.514 (<0.001)**	0.323 (0.005)	**0.547 (<0.001)**	**-0.654 (<0.001)**
Soil Cr	**-0.487 (<0.001)**	-0.156 (0.188)	**0.398 (<0.001)**	**0.414 (<0.001)**	**0.434 (<0.001)**	**0.402 (<0.001)**	-0.325 (0.005)
Plant Al	-0.023 (0.850)	0.024 (0.842)	-0.034 (0.776)	0.02 (0.869)	-0.008 (0.948)	-0.061 (0.610)	0.148 (0.213)
Plant B	-0.081 (0.496)	-0.135 (0.258)	0.103 (0.390)	0.198 (0.096)	0.018 (0.881)	0.079 (0.506)	0.05 (0.675)
Plant Ca	-0.29 (0.013)	**-0.512 (<0.001)**	0.31 (0.008)	**0.547 (<0.001)**	0.208 (0.079)	**0.52 (<0.001)**	-0.354 (0.002)
Plant Cd	0.165 (0.166)	0.022 (0.851)	-0.207 (0.081)	-0.208 (0.079)	-0.06 (0.615)	-0.163 (0.170)	0.176 (0.137)
Plant Cr	**-0.412 (<0.001)**	-0.114 (0.341)	0.322 (0.005)	**0.399 (<0.001)**	0.183 (0.123)	**0.434 (<0.001)**	**-0.407 (<0.001)**
Plant Cu	-0.11 (0.356)	0.236 (0.045)	-0.179 (0.132)	-0.104 (0.384)	0.035 (0.767)	-0.117 (0.327)	0.05 (0.672)
Plant Fe	-0.131 (0.273)	-0.115 (0.337)	0.055 (0.647)	0.168 (0.157)	0.093 (0.436)	0.104 (0.383)	0.012 (0.918)
Plant K	-0.067 (0.576)	0.017 (0.886)	-0.154 (0.196)	-0.018 (0.883)	0.098 (0.413)	-0.048 (0.688)	0.114 (0.341)
Plant Mg	**0.622 (<0.001)**	0.087 (0.466)	**-0.657 (<0.001)**	**-0.468 (<0.001)**	**-0.611 (<0.001)**	**-0.427 (<0.001)**	**0.387 (<0.001)**
Plant Mn	-0.256 (0.030)	-0.046 (0.703)	0.07 (0.560)	0.1 (0.400)	0.238 (0.044)	0.105 (0.376)	0.01 (0.934)
Plant Na	-0.023 (0.845)	0.038 (0.749)	0.122 (0.306)	0.107 (0.370)	-0.077 (0.521)	0.266 (0.024)	**-0.366 (0.001)**
Plant Ni	**-0.508 (<0.001)**	-0.229 (0.052)	**0.481 (<0.001)**	**0.573 (<0.001)**	**0.457 (<0.001)**	**0.639 (<0.001)**	**-0.508 (<0.001)**
Plant P	-0.002 (0.989)	-0.207 (0.080)	-0.032 (0.786)	0.228 (0.054)	-0.023 (0.847)	0.292 (0.013)	-0.283 (0.016)
Plant Pb	**-0.424 (<0.001)**	-0.336 (0.004)	**0.43 (<0.001)**	**0.606 (<0.001)**	**0.373 (0.001)**	**0.609 (<0.001)**	**-0.423 (<0.001)**
Plant Zn	0.228 (0.054)	0.131 (0.270)	-0.358 (0.002)	-0.131 (0.273)	-0.301 (0.010)	-0.015 (0.901)	0.004 (0.975)

Table 3-7. Spearman correlation coefficients (r) for plant and soil parameters describing relative abundance of major bacterial orders in 2015. *P* values are shown in parenthesis and significant values, based on Bonferroni adjustment for multiple comparisons, are bolded.

Plant / soil parameter	*Actinomycetales*	*Burkholderiales*	*Acidobacteria Gp. 4*	*Acidobacteria Gp. 1*	*Pseudomonadales*
Biomass Yield	0.07 (0.586)	0.366 (0.003)	-0.219 (0.087)	0.271 (0.033)	0.233 (0.068)
Root Biomass	0.19 (0.138)	0.113 (0.378)	-0.122 (0.342)	0.26 (0.041)	0.075 (0.559)
Soil Moisture	-0.326 (0.009)	-0.286 (0.024)	**0.517 (<0.001)**	-0.336 (0.007)	-0.311 (0.014)
pH	-0.285 (0.025)	-0.383 (0.002)	**0.624 (<0.001)**	**-0.624 (<0.001)**	-0.003 (0.982)
Soil NO_3^-	0.124 (0.334)	-0.254 (0.046)	0.088 (0.493)	-0.277 (0.029)	-0.119 (0.356)
Soil NH_4^+	-0.042 (0.745)	0.117 (0.365)	-0.084 (0.517)	0.047 (0.718)	-0.152 (0.238)
Plant Al	-0.346 (0.006)	**-0.6 (<0.001)**	**0.43 (<0.001)**	-0.383 (0.002)	0.094 (0.467)
Plant B	-0.012 (0.926)	-0.351 (0.005)	0.387 (0.002)	**-0.414 (<0.001)**	0.024 (0.849)
Plant Ca	0.004 (0.976)	-0.244 (0.056)	0.231 (0.071)	-0.192 (0.135)	-0.007 (0.953)
Plant Cd	0.012 (0.926)	-0.205 (0.110)	-0.03 (0.813)	-0.06 (0.640)	-0.218 (0.089)
Plant Cr	-0.144 (0.264)	-0.297 (0.019)	0.076 (0.557)	-0.134 (0.297)	-0.016 (0.902)
Plant Cu	-0.13 (0.311)	-0.314 (0.013)	-0.12 (0.351)	-0.029 (0.821)	0.138 (0.285)
Plant Fe	-0.182 (0.155)	**-0.473 (<0.001)**	0.31 (0.014)	-0.295 (0.020)	0.175 (0.172)
Plant K	0.102 (0.430)	-0.235 (0.066)	0.245 (0.055)	-0.373 (0.002)	-0.145 (0.258)
Plant Mg	-0.055 (0.670)	-0.138 (0.284)	0.267 (0.036)	-0.187 (0.145)	0.026 (0.840)
Plant Mn	0.191 (0.136)	0.089 (0.491)	**-0.562 (<0.001)**	**0.513 (<0.001)**	-0.017 (0.894)
Plant Na	0.101 (0.435)	0.018 (0.887)	-0.121 (0.348)	-0.035 (0.786)	0.208 (0.104)
Plant Ni	0.1 (0.440)	-0.163 (0.204)	-0.145 (0.259)	0.009 (0.945)	-0.16 (0.213)
Plant P	-0.112 (0.387)	-0.331 (0.008)	0.351 (0.005)	**-0.493 (<0.001)**	0.005 (0.971)
Plant Pb	-0.064 (0.620)	-0.339 (0.007)	0.391 (0.002)	**-0.431 (<0.001)**	-0.134 (0.299)
Plant Zn	-0.08 (0.533)	-0.281 (0.027)	0.055 (0.669)	-0.193 (0.133)	0.14 (0.275)

Table 3-8. Spearman correlation coefficients (r) for plant and soil parameters describing relative abundance of major fungal species in 2014. *P* values are shown in parenthesis and significant values, based on Bonferroni adjustment for multiple comparisons, are bolded.

Plant / soil parameters	*Cryptococcus terreus*	*Fusarium fujikuroi*	*Fusarium sp. BS 8*	*Phallus rugulosus*
Biomass yield	-0.061 (0.614)	-0.253 (0.033)	-0.021 (0.863)	0.02 (0.869)
Root biomass	-0.264 (0.026)	0.008 (0.949)	0.108 (0.370)	0.096 (0.424)
Soil moisture	-0.188 (0.115)	-0.137 (0.255)	-0.044 (0.717)	0.364 (0.002)
Soil P	-0.364 (0.002)	0.132 (0.271)	0.323 (0.006)	0.291 (0.014)
Soil Fe	**-0.453 (<0.001)**	-0.315 (0.007)	0.085 (0.478)	**0.444 (<0.001)**
Soil Mn	0.08 (0.504)	-0.267 (0.024)	-0.071 (0.555)	-0.203 (0.089)
Soil Zn	**-0.489 (<0.001)**	-0.07 (0.558)	0.181 (0.130)	**0.566 (<0.001)**
Soil Cu	-0.207 (0.082)	-0.025 (0.836)	0.083 (0.492)	0.161 (0.179)
Soil Pb	-0.31 (0.008)	-0.318 (0.007)	0.134 (0.265)	0.266 (0.024)
Soil Ni	-0.149 (0.215)	-0.247 (0.038)	-0.166 (0.165)	0.274 (0.021)
Soil Cd	-0.228 (0.055)	0.069 (0.568)	0.02 (0.870)	**0.466 (<0.001)**
Soil Cr	-0.349 (0.003)	-0.27 (0.023)	0.064 (0.593)	**0.506 (<0.001)**
Plant Al	-0.021 (0.858)	-0.152 (0.205)	-0.047 (0.697)	-0.037 (0.756)
Plant B	0.145 (0.226)	0.053 (0.659)	0.009 (0.942)	-0.035 (0.772)
Plant Ca	-0.197 (0.099)	0.072 (0.548)	0.041 (0.732)	**0.408 (<0.001)**
Plant Cd	0.124 (0.301)	-0.164 (0.170)	-0.083 (0.489)	-0.174 (0.147)
Plant Cr	-0.183 (0.126)	-0.065 (0.589)	0.042 (0.725)	0.31 (0.008)
Plant Cu	-0.206 (0.084)	-0.187 (0.117)	0.228 (0.056)	0.071 (0.556)
Plant Fe	-0.145 (0.227)	-0.138 (0.251)	0.062 (0.605)	0.117 (0.329)
Plant K	-0.303 (0.010)	-0.287 (0.015)	0.26 (0.029)	0.2 (0.093)
Plant Mg	0.314 (0.007)	0.248 (0.037)	0.135 (0.261)	**-0.428 (0<0.00)**
Plant Mn	**-0.407 (<0.001)**	-0.364 (0.002)	0.021 (0.860)	0.313 (0.008)
Plant Na	-0.008 (0.945)	0.018 (0.879)	-0.148 (0.217)	0.045 (0.709)
Plant Ni	**-0.589 (<0.001)**	-0.165 (0.169)	0.171 (0.152)	**0.493 (<0.001)**
Plant P	-0.204 (0.088)	0.04 (0.740)	0.308 (0.009)	0.246 (0.038)
Plant Pb	-0.332 (0.004)	0.049 (0.684)	0.235 (0.048)	**0.381 (0.001)**
Plant Zn	-0.046 (0.701)	0.087 (0.471)	0.314 (0.007)	-0.215 (0.071)

Table 3-9. Spearman correlation coefficients (r) for plant and soil parameters describing relative abundance of major fungal species in 2015. *P* values are shown in parenthesis and significant values, based on Bonferroni adjustment for multiple comparisons, are bolded.

Plant / soil parameter	*Fusarium fujikuroi*	*Fusarium sp. BS 8*	*Ascomycota sp FL 2010c*	*Fusicolla violacea*	*Atheliaceae sp.*	*Entoloma sp. XLH 2013b*	*Ramaria coulterae*
Biomass Yield	-0.197 (0.096)	-0.358 (0.002)	0.124 (0.299)	0.241 (0.041)	**0.424 (<0.001)**	-0.041 (0.731)	0.23 (0.052)
Root Biomass	0.07 (0.556)	-0.143 (0.231)	0.107 (0.371)	0.155 (0.193)	0.213 (0.073)	-0.06 (0.616)	0.188 (0.113)
Soil Moisture	**0.393 (<0.001)**	0.193 (0.103)	-0.131 (0.271)	**-0.398 (<0.001)**	-0.164 (0.167)	0.297 (0.011)	-0.219 (0.064)
pH	**0.366 (0.001)**	0.16 (0.180)	-0.068 (0.572)	-0.156 (0.191)	-0.358 (0.002)	0.262 (0.026)	-0.231 (0.051)
Soil NO_3^-	0.167 (0.159)	0.111 (0.354)	0.047 (0.696)	-0.135 (0.258)	**-0.373 (0.001)**	0.109 (0.359)	-0.226 (0.056)
Soil NH_4^+	0.047 (0.695)	0.115 (0.335)	0.154 (0.196)	0.092 (0.443)	0.066 (0.578)	0.117 (0.327)	0.115 (0.335)
Plant Al	0.243 (0.039)	0.106 (0.373)	-0.357 (0.002)	**-0.396 (<0.001)**	**-0.442 (<0.001)**	0.081 (0.498)	-0.23 (0.052)
Plant B	0.319 (0.006)	0.013 (0.911)	-0.098 (0.412)	-0.16 (0.180)	-0.238 (0.044)	0.013 (0.914)	-0.313 (0.007)
Plant Ca	0.018 (0.878)	-0.065 (0.588)	0.045 (0.705)	-0.134 (0.259)	0.047 (0.691)	0.128 (0.281)	0.248 (0.035)
Plant Cd	0.272 (0.021)	**0.391 (<0.001)**	-0.242 (0.041)	-0.29 (0.013)	-0.286 (0.015)	-0.168 (0.157)	-0.19 (0.108)
Plant Cr	-0.065 (0.586)	0.101 (0.399)	-0.116 (0.333)	-0.108 (0.365)	-0.23 (0.052)	0.115 (0.333)	0.065 (0.587)
Plant Cu	0.078 (0.514)	0.01 (0.931)	-0.332 (0.004)	-0.307 (0.008)	-0.051 (0.668)	-0.24 (0.042)	0.003 (0.982)
Plant Fe	0.109 (0.360)	-0.023 (0.844)	-0.35 (0.002)	-0.354 (0.002)	-0.234 (0.047)	0.103 (0.390)	-0.082 (0.493)
Plant K	0.22 (0.063)	0.068 (0.568)	-0.012 (0.918)	-0.062 (0.606)	-0.3 (0.010)	0.031 (0.796)	-0.327 (0.005)
Plant Mg	-0.113 (0.345)	-0.143 (0.231)	0.114 (0.337)	0.159 (0.181)	-0.009 (0.942)	0.323 (0.005)	0.172 (0.148)
Plant Mn	-0.073 (0.543)	0.037 (0.760)	-0.078 (0.512)	-0.081 (0.497)	0.12 (0.315)	**-0.417 (<0.001)**	0.172 (0.148)
Plant Na	-0.201 (0.090)	-0.222 (0.060)	0.021 (0.860)	0.347 (0.002)	0.067 (0.574)	-0.173 (0.144)	0.125 (0.293)
Plant Ni	0.029 (0.811)	0.082 (0.492)	-0.036 (0.760)	-0.004 (0.976)	-0.204 (0.086)	-0.087 (0.468)	0.058 (0.629)
Plant P	0.22 (0.063)	-0.074 (0.534)	-0.064 (0.591)	-0.019 (0.875)	-0.002 (0.987)	0.103 (0.389)	-0.038 (0.752)
Plant Pb	0.254 (0.031)	**0.367 (0.001)**	-0.133 (0.263)	**-0.478 (<0.001)**	-0.284 (0.015)	0.078 (0.512)	-0.237 (0.045)
Plant Zn	0.194 (0.102)	0.122 (0.305)	-0.29 (0.013)	-0.108 (0.364)	-0.072 (0.545)	-0.054 (0.651)	-0.011 (0.925)

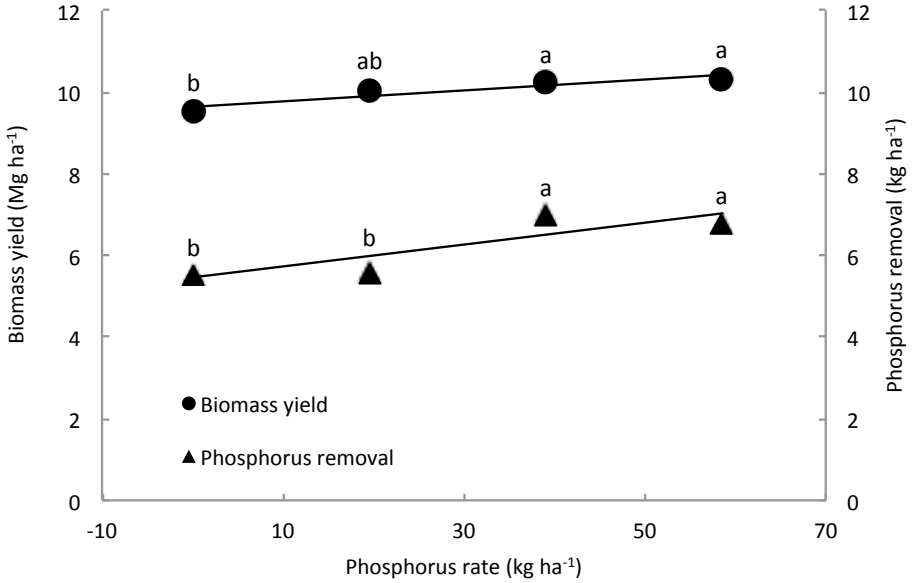


Figure 3-1. Biomass yields (2014-2016) and P removal (2014-2015) in switchgrass, averaged over production year and cultivar, as a function of increasing P rate at Lamberton, MN. The regression equation for yield is $Y = 0.0135X + 9.6386$, $r^2 = 0.83$, root mean square error (RMSE) = 0.2, and for P removal is $Y = 0.0268X + 5.4615$, $r^2 = 0.74$, RMSE = 0.5.

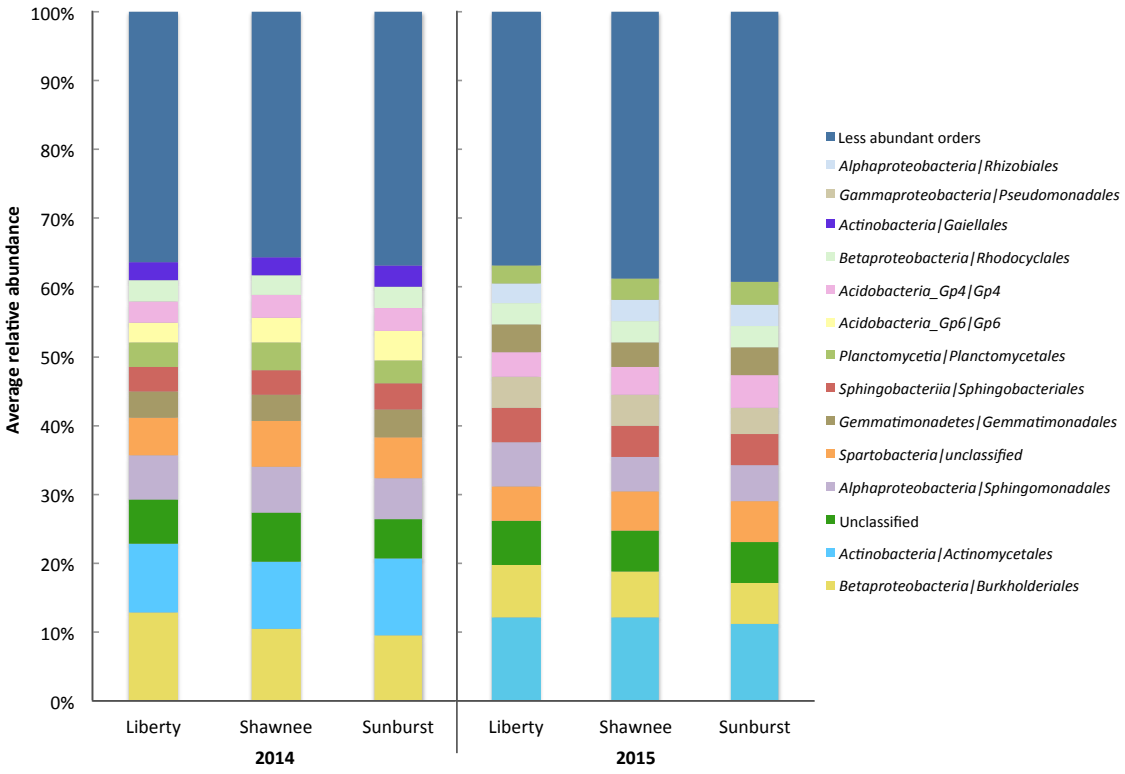


Figure 3-2. Distribution of abundant bacterial orders found in rhizosphere soil samples from three switchgrass cultivars collected in 2014 (n=72) and 2015 (n=62) at Lamberton, MN.

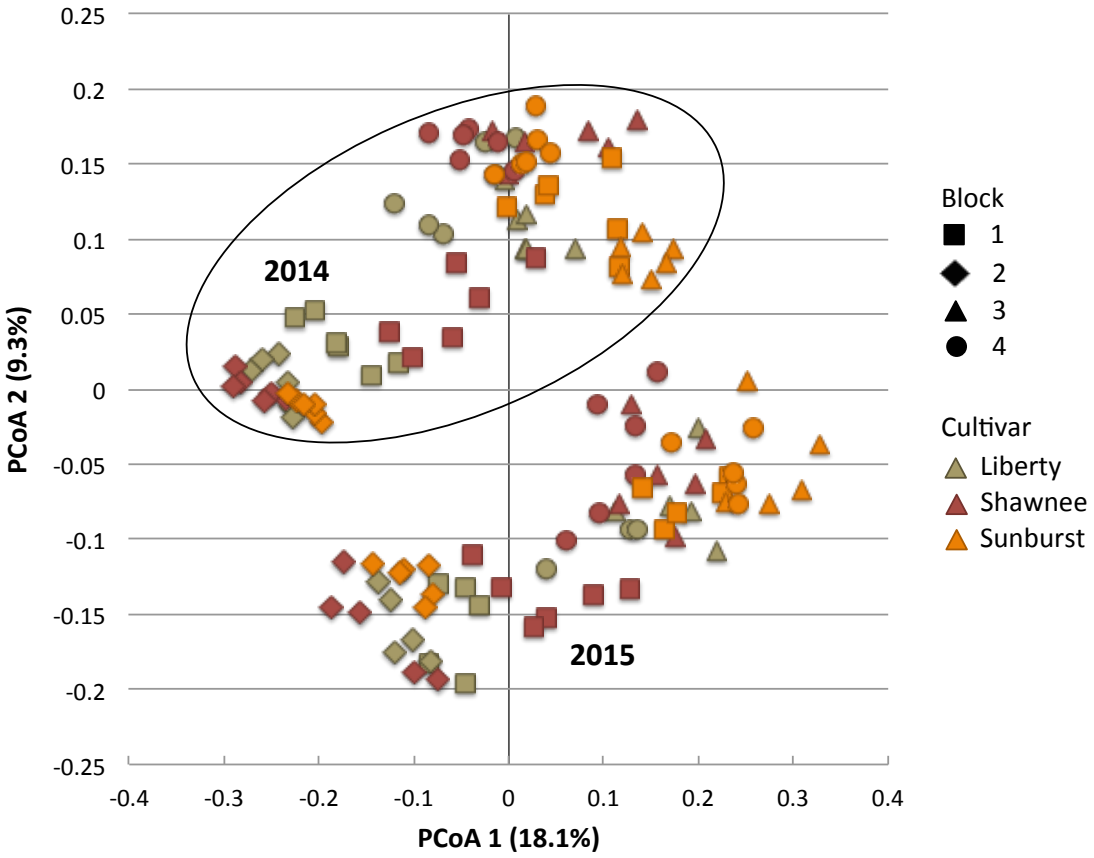


Figure 3-3. Principal coordinate analysis of rhizosphere bacterial samples based on Bray-Curtis dissimilarity distances. The r^2 value relating ordination to the distance matrix is 0.72.

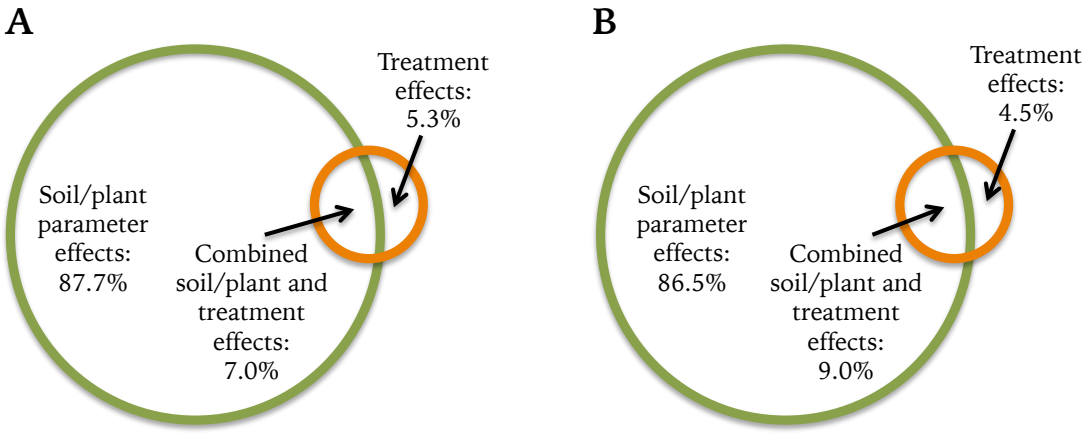
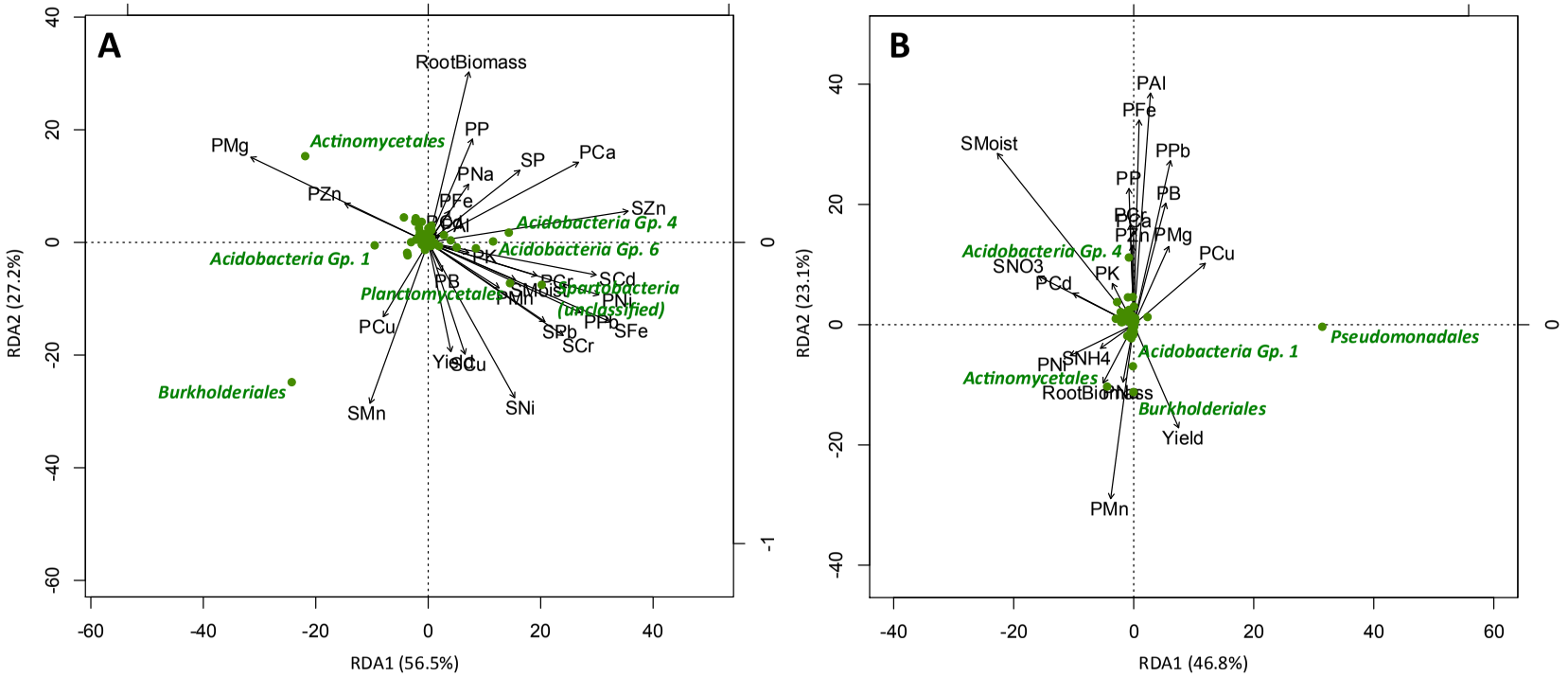


Figure 3-4. Variance partitioning of relative abundance of bacterial orders as a function of soil and plant physiochemical parameters, treatment effects, and combined soil/plant and treatment effects in 2014 (A) and 2015 (B).

Figure 3-5. Redundancy analysis showing variance in relative abundances of bacterial orders as explained by plant and soil parameters. A) 2014, n=72 samples, and B) 2015, n=62 samples.



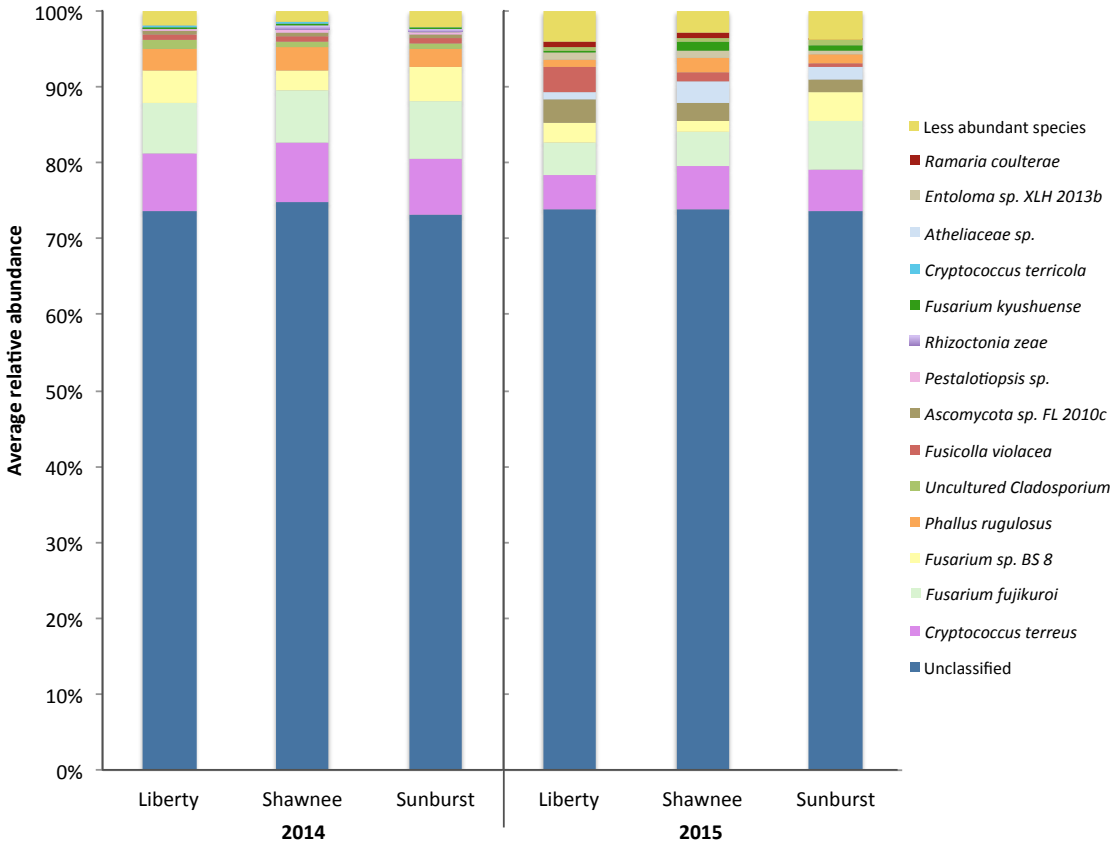


Figure 3-6. Distribution of abundant fungal species found in rhizosphere soil samples from three switchgrass cultivars collected in 2014 (n=71) and 2015 (n=72) at Lamberton, MN.

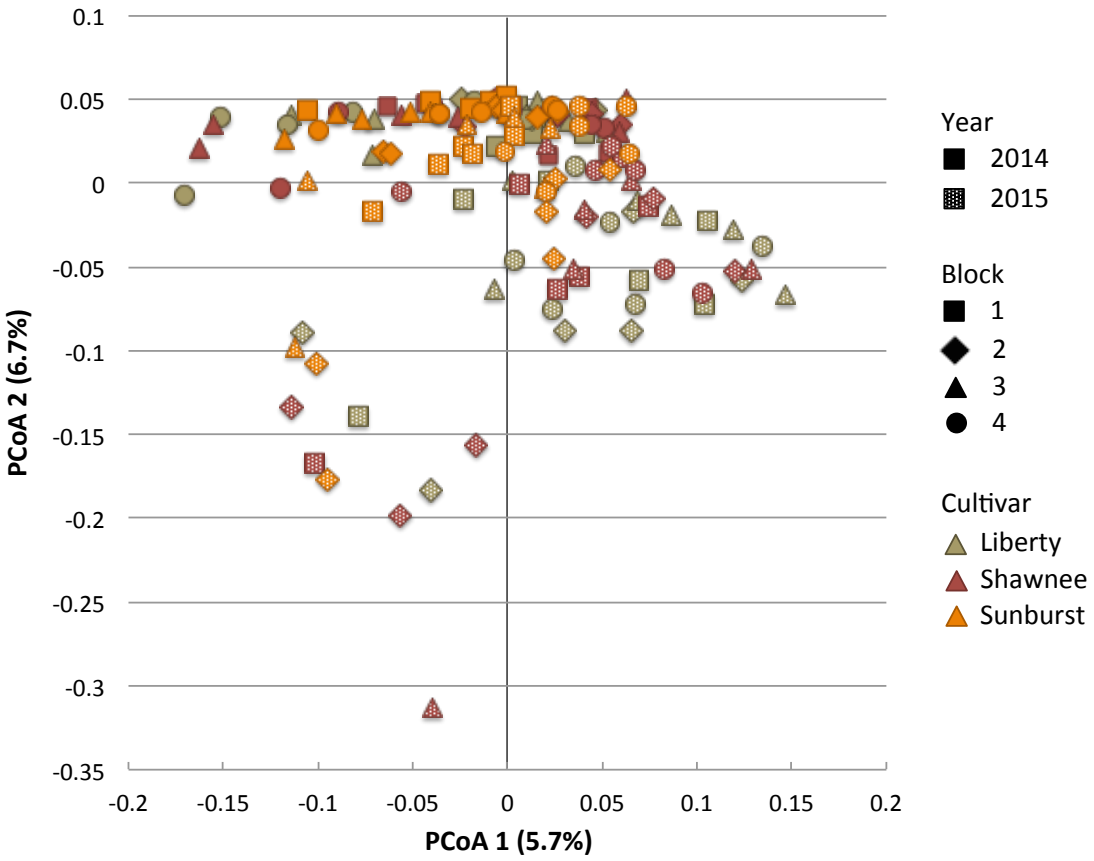


Figure 3-7. Principal coordinate analysis of rhizosphere fungal samples based on Bray-Curtis dissimilarity distances. The r^2 value relating ordination to the Bray-Curtis distance matrix is 0.76.

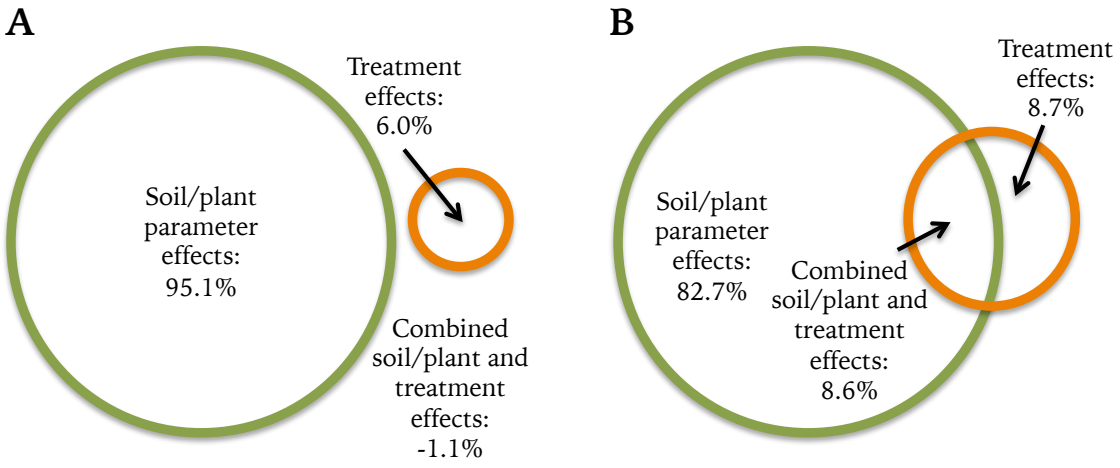
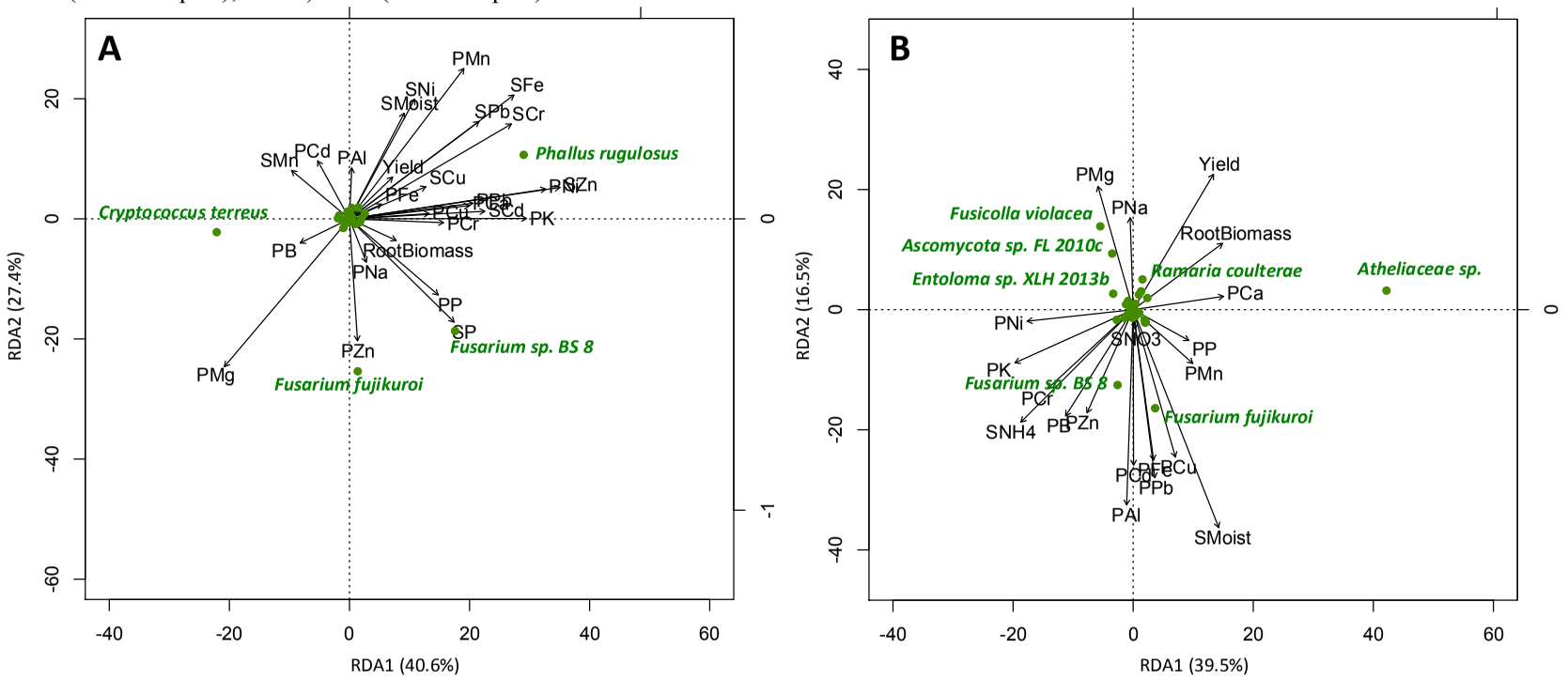


Figure 3-8. Variance partitioning of relative abundance of fungal species as a function of soil and plant physiochemical parameters, treatment effects, and combined soil/plant and treatment effects in 2014 (A) and 2015 (B).

Figure 3-9. Redundancy analysis showing variance in relative abundances of fungal species as explained by plant and soil parameters. A) 2014 (n=71 samples), and B) 2015 (n=72 samples).



Conclusions

Overall, results from this study greatly enhanced regional knowledge of best management practices for switchgrass and mixed native perennials grown for bioenergy. With respect to N fertility, maximum biomass production can be achieved with either a well-adapted switchgrass variety or low diversity grass mix fertilized with 56 kg N ha^{-1} annually, post-establishment, on a moderately-productive loam soil, and with 112 kg N ha^{-1} annually on an excessively-drained sandy loam soil (Chapter 1). With respect to P fertility, biomass increased linearly in response to P applied prior to establishment on a clay loam soil testing at low or medium phosphorus levels (Bray-P1) (Chapter 2), although further work is necessary to clarify best management practices. While producers may have flexibility in harvest timing for some feedstocks in the first few years following establishment, a post-frost harvest regime will remove fewer nutrients and promote stand longevity with fewer inputs over time.

These results also address the complexity of the cultivar × environment interaction in switchgrass production. For example, "Sunburst" switchgrass produced dry matter yields comparable to 'Shawnee' and greater than 'Liberty' in the N study (Chapter 1) at both Becker and Lamberton, but 'Sunburst' yields were less than those in both 'Shawnee' and 'Liberty' in the P study (Chapter 2). The N and P study locations at Lamberton were located within 5 km of each other, on similar (loam-clay loam) soils, but the P study was established one year later.

Results from this study highlight the extreme diversity of soil microbes in the switchgrass rhizosphere and underscore the importance of preexisting soil characteristics

and environment in shaping the microbial community. Management decisions, however, also influenced the microbial rhizosphere community, creating differences in community structure as a function of cultivar in both bacterial (Chapter 2) and fungal (Chapters 2 and 3) populations. Differences as a function of cultivar were not persistent across similar microbial taxa at multiple locations, however. N fertilization treatment did produce consistent differences in bacterial orders across locations, including those involved in N dynamics in soil: *Nitrosomonadales* and *Rhodocyclales* (Chapter 2). Phosphorus fertilization did not affect fungal or bacterial community structure in the switchgrass rhizosphere (Chapter 3). Future advances in sequencing technology, bioinformatics, and fungal taxonomic databases may be necessary to better address fungal community dynamics in the rhizosphere.

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Appendix A

```
* This code will analyze dry matter yield data from Becker, summed over three post-establishment
years. The experimental design was a split, split-plot in four replications, with harvest treatment
('harvest' in model) as main plot, cultivar/perennial mix ('grass' in model) as subplot, and nitrogen
application rate ('nrate') as sub-subplot. Replication ('rep') was treated as random, all other effects
were fixed.;

*Files must be Windows-format .csv for import;

proc import datafile="C:\SASFiles\FACTB\FACTB_all_DM_SUM.csv" out=mydata   dbms=dlm
replace;
delimiter=',';
getnames=yes;
run;

options nodate ps=60 ls=80;

proc sort;
     by harvest grass nrate;

data mydata;
     set mydata;    *Options, if any, go here;

ods graphics on;

run;

proc mixed data=mydata;    *Use REML default;
     class rep harvest grass nrate;
     model sum_yield = harvest | grass | nrate / residual ddfm=satterthwaite;    * Using '|' tells SAS to
                    model all of the interactions between factors, Satterthwaite gives correct standard
                    errors in split-plots;
     parms /nobound;  *Prevents error pooling;
     random rep
          rep*harvest
          rep*harvest*grass;
     lsmeans harvest*grass*nrate /pdiff;

     store sasuser.FACTBDM1;    *This stores the lsmeans data for use in proc PLM, below;

run;

proc PLM restore=sasuser.FACTBDM1;

     lsmeans harvest*grass*nrate / lines;      *The 'lines' command produces mean separation,
designated using letters;

run;

ods graphics off;
```

Appendix B

```
* This code will analyze dry matter yield data from Lamberton over three years (including
establishment year). The experimental design was a split-plot in four replications, with cultivar
('feedstock' in model) as main plot and phosphorus application rate ('prate') as subplot. Year was
treated as a sub-subplot in the model, after Steel and Torrie (1997). Replication ('rep') was treated as
random, all other effects were fixed.;

*TO IMPORT FILES: Must be in Windows-format .csv

proc import datafile="C:\SASFiles\SNAP\SNAP_harvest_by_year.csv" out=mydata   dbms=dlm
replace;
delimiter=',';
 getnames=yes;
run;
options nodate ps=60 ls=80;

proc sort;
     by year feedstock prate rep;

data mydata;
     set mydata;     *Options, if any, go here;

ods graphics on;

proc mixed data=mydata;
     class rep year feedstock prate;
     model yield = feedstock | prate | year / residual ddfm=satterthwaite;   * Using '|' tells SAS to model
                    all of the interactions between factors, Satterthwaite gives correct standard errors in
                    split-plots;
     random rep
          rep*feedstock
          rep*feedstock*prate;
     parms /nobound;     *Prevents error pooling;
     lsmeans prate feedstock year*feedstock /pdiff;
     contrast 'Linear' prate -3 -1 1 3;
     contrast 'Quadratic' prate 1 -1 -1 1;
     contrast 'Cubic' prate -1 3 -3 1;

     store sasuser.FACTBEST1;    *This stores the lsmeans data for use in proc PLM, below;

proc PLM restore=sasuser.FACTBEST1;

     lsmeans year*feedstock / lines;    *The 'lines' command produces mean separation, designated
using letters;

run;

ods graphics off;
```